

**OESTROGEN CONTROL
OF GONADOTROPHIN SECRETION
IN THE IMMATURE FEMALE RAT**

In vitro and in vivo studies

THESE

présentée à la Faculté de médecine de l'Université de Genève
pour obtenir le grade de docteur en médecine

par

Dominique DE ZIEGLER

de

Genève

Thèse No 3533

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Editions Médecine et Hygiène
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La Faculté de médecine, sur le préavis du professeur Kurt B. Ruf, directeur de thèse, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

Genève, le 21 septembre 1976

Le doyen :
Marcel Jenny

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Ce travail a été effectué au sein du Département de physiologie, Ecole de médecine, Université de Genève, dirigé par le professeur Jean Posternak.

Que le professeur Posternak trouve ici l'expression de ma sincère gratitude et de mon respectueux attachement.

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- III. Anterior pituitary sensitivity in immature female rats stimulated in vivo with gonadotrophin releasing hormone : effect of priming with oestrogen, pregnant mare serum, gonadotrophin, or Brain Lesion.

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I. INTRODUCTION

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Preliminary remark :

The work reported in this study is concerned exclusively with the female rat. Comparisons with conditions known to exist in man are made as often as possible during discussions.

1. Steroidal control of gonadotrophin secretion

The control exerted by gonadal steroids on adeno-hypophysial secretion of gonadotrophins has been intensively studied in the past five decades. It is now known that in the adult female, oestrogen controls gonadotrophin secretion in either a facilitatory or an inhibitory fashion (1).

We shall consider these two modes of control successively, together with the location of the structures via which they are mediated. The ontogeny of these effects will also be shortly reviewed before introducing the particular contribution of this thesis.

- Negative feedback

From the early studies of Moore and Price (2) and Hohlweg (3) the negative feedback properties of oestrogen on gonadotrophin secretion were recognized. These authors reported that treatment with exogenous oestradiol benzoate (OB) would lead to the disappearance of the morphological signs of hyperfunction normally seen in the pituitary after ovariectomy (i.e. castration cells) (for reviews, see 1, 4). The development of sensitive and precise gonadotrophin assays allowed studies to be made on the negative feedback of oestrogen under experimental conditions. In particular, studies were carried out on the rise in plasma and pituitary gonadotrophin observed after gonadectomy as well as on the nature and the amount of exogenous treatment required to prevent this postcastration rise in gonadotrophins. Effects produced by small physiological doses of oestrogen replacement therapy on post-castration gonadotrophin levels had already been studied with LH and FSH bioassays. Plasma gonadotrophins were evaluated with the ovarian ascorbic acid depletion assay (OAA) for LH (5) and with the HCG augmentation assay for FSH (6). These results indicated that in the female rat oestrogen exerts a negative feedback on both the synthesis and release of LH and FSH as illustrated by a decrease of both the gonadotrophin plasma level and pituitary content after OB replacement. These early results have now been extensively confirmed with the development of gonadotrophin radioimmunoassays (RIA) (7, 8). For references on the development of rat gonadotrophin RIA, see 9, 10 ; for human gonadotrophins, see 11, 12. In contrast to the negative feedback effect exerted by oestrogen another major gonadal steroid, progesterone, appears to be inert with respect to the control of tonic gonadotrophin secretion (rodents : 8 ; monkeys : 13). Nevertheless, progesterone affects gonadotrophin secretion by modulating the positive feedback of oestrogen ; this effect of progesterone is discussed on page 6.

- Positive feedback

Oestrogens and progestins have been known for a long time to exert a stimulatory effect on ovulation. In the adult female, oestrogen has been implicated

in the triggering of the preovulatory rise in plasma gonadotrophin, particularly the preovulatory LH peak. This property, referred to as the positive feedback of oestrogen on gonadotrophin release, was reported by Everett (14). In these early studies, he observed that exogenous oestrogen could advance ovulation by one day when oestradiol benzoate was given, on dioestrus II, to 5-day cycling adult female rats (see appendix 1 on reproductive cycle of female rats). The development of radioimmunoassay methods has allowed simultaneous determination of plasma oestrogen and gonadotrophin levels in many different animal species and in particular throughout the oestrous cycle of the rat (Fig. 1) (15, 16, 17, 18) and during the human menstrual cycle (19). Plasma oestrogen has been found to increase during the follicular phase of the reproductive cycle to a peak which, in all mammals invariably precedes the preovulatory LH surge and has thus been considered to play a causal role in the genesis of this surge (18, 19, 20). Many studies have tried to clarify the role played by oestrogen in the preovulatory rise of gonadotrophin levels focusing on the consequences of oestrogen withdrawal. This has been achieved by means of 'anti-oestrogen' products which can be classified in two different groups : in the first "anti-oestrogen" category are the pharmaceutical drugs, with clomiphene being their most typical representative ; these drugs interfere with oestrogen uptake by target organs probably by competing at the receptor sites (21). Ovulation is prevented when the anti-oestrogen is given to female rats on the afternoon of the day preceding pro-oestrus i.e. at the time when plasma oestrogen starts to rise (22, 23, 24). Comparable results have been obtained with another type of "anti-oestrogen" i.e. specific antisera to oestrogen. This immunological approach excludes a non-specific effect of the anti-oestrogen drug : ewes immunised with conjugates of 17β -oestradiol hemi-succinate produce antibodies capable of blocking the biological effect of endogenous and exogenous 17β -oestradiol (25) which prevent the pro-oestrus gonadotrophin surge and the ovulation from occurring when given to dioestrous female rats (26). Unlike the drugs competing with oestrogen at receptor sites, these specific antibodies probably neutralize endogenous hormone in peripheral blood (25). Positive feedback of oestrogen has been demonstrated in the female of many other species and particularly in primates (27 ; for review see 28) and in humans (29, 30).

Progesterone has also been shown to facilitate ovulation under certain circumstances and to abolish oestrogen positive feedback in others. Progesterone advances the preovulatory LH surge by one day when given to 5-day cycling female rats at noon on the third day of dioestrus (1, 14) or induces an LH surge within 6 hours in ovariectomised, oestrogen-primed, female rats (31). This short-term facilitatory effect of progesterone on gonadotrophin secretion is exerted only after a previous priming of the hypothalamo-pituitary axis with oestrogen (8, 32, 33). On the other hand progesterone blocks ovulation when given at 2200 h on dioestrus II in 4-day cycling adult female rats by interfering with the pro-oestrous oestrogen rise (20, 34). Recent studies have shown that progesterone induces, after a four to six hours delay, a rise in gonadotrophin releasing hormone (GnRH) content of pituitary stalk blood in ovariectomized oestrogen-primed female rats (35). This observation tends thus to incriminate the hypothalamus as the site mediating the facilitatory effect of progesterone.

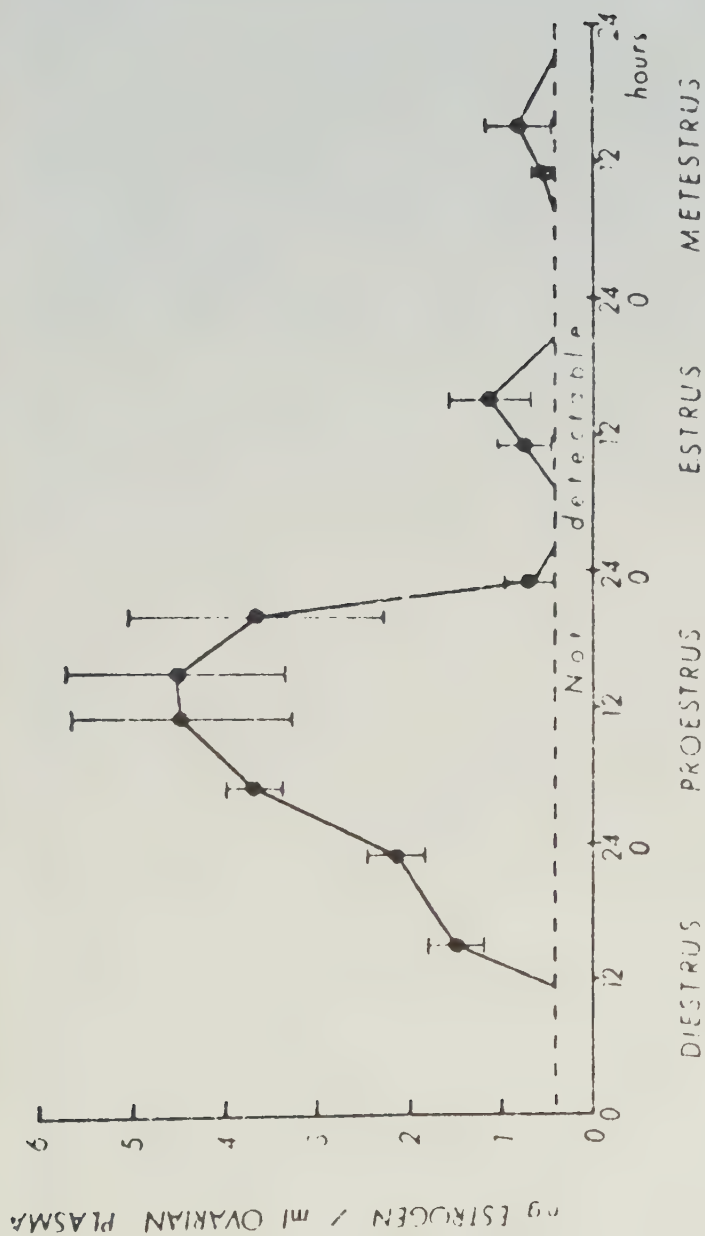


Fig. 1
Oestrogen concentration in ovarian venous plasma during the rat oestrous cycle, each value is the mean of 2-8 determinations plotted $\pm$ SE.

from Yoshinaga et al, 1969 (15)

Many experimental models have been developed to investigate the facilitatory effect of ovarian steroids on gonadotrophin release. Two of them have been extensively documented in the female rat : the first model uses long-term ovariectomised (approximately 8 weeks) oestrogen-primed (10-20 μ g OB) adult female rats ; injection of either oestrogen (10-20 μ g) or progesterone (1 mg) 48 or 72 h respectively after the first oestrogen priming injection will trigger an LH peak of preovulatory magnitude within respectively 30 and 6 h (31, 35). An extension of this model not requiring ovariectomy has been developed to study positive feedback in immature female rats (36) (see section 3 of chapter I). In another schema, Ramirez and Sawyer (37) have used short-term (48 h) ovariectomised female rats to study the stimulatory action of oestrogen on LH secretion. This model, based on the delay observed in the post-castration rise of LH, offers the advantage of being simpler by not requiring a first priming injection of OB. Advantages and draw-backs of these and other documented experimental models as highly reproducible manipulations for studying oestrogen feedback are discussed in a recent review by Brown-Grant (38).

2. Location of the sites of steroid feedback control over gonadotrophin secretion.

In trying to locate the anatomical site of steroid feedback action the question arises whether there is one or more possible site(s) for the mediation of inhibitory and stimulatory feedback mechanisms and whether these sites are more likely to be found at the level of the brain or pituitary. Also, it is legitimate to ask whether ovarian steroids exert positive or negative feedback control at the same places and at the same or at different times. In an early hypothesis on the feedback control exerted by oestrogen on gonadotrophin production, Moore and Price (2) located the action of oestrogen at the anterior pituitary level.

Thereafter, however, interest in feedback mechanisms shifted to the hypothalamus, mainly after publication of the impressive work of Harris (39) in which he confirmed his early theory of anterior pituitary control exerted by hypothalamic chemotransmitters (40,41). The rationale behind early investigations was that sex steroids may act at the sites of production of the releasing hormones. Attempts were made to localize the site of production of hypophysiotropic hormones and in particular the GnRH production site.

- Pituitary grafts

One method used to locate the site of releasing hormone synthesis is based on the histological appearance of anterior pituitary grafts implanted into various hypothalamic and extrahypothalamic regions of the brain ; the structural integrity of the transplants was maintained only when in the presence of releasing factors. Using this technique, Halász (42) demonstrated that anterior pituitary grafts preserved their morphology as well as their function only when the gland fragments were grafted into the medial basal hypothalamus. This hypothalamic region, referred to as the "hypophysiotrophic area", includes the arcuate nucleus, the ventral part of the anterior periventricular nuclei, the medial part of the retrochiasmatic region and the median eminence (for review, see Halász, 1972, ref. 43).

- Brain lesions and brain stimulations

This approach is based on electrical stimulation or lesioning of different brain areas. Various effects were observed, according to electrode placement. Destructive brain lesions performed in the medial basal hypothalamus abolish negative feedback control of oestrogen (44,45) whereas rostral lesions located in the preoptic area (POA) evoke vaginal cornification together with failure to ovulate (1, 46). In this latter case, ovaries are filled with large follicles reflecting intense oestrogen production and the tonic secretion of gonadotrophin is maintained (46) ; the negative feedback action of gonadal steroids is preserved (44, 46) although no surge in LH is observed (46). On the other hand, electrical stimulation of the medial POA or of the median eminence in adult female rats results in an increase in plasma gonadotrophins (47,48,49) or pituitary stalk blood content of GnRH (50).

- Surgical isolation

Further light has been shed on the nervous control of pituitary function by surgical isolation or partial deafferentation of the hypophysiotrophic area from the rest of the brain with the use of a specially designed knife blade held in a stereotaxic apparatus (51). Surgical disconnection of the hypothalamic hypophysiotrophic area from the rest of the brain does not produce any modification of gonadotrophin secretion in the male, but it interferes in the female with the cyclic LH peak and ovulation : the ovaries are not atrophic and show significant follicular development though fresh corpora lutea are not seen. It appears that surgical isolation of the basal hypothalamus does not alter the tonic secretion of gonadotrophins but, in rats, suppresses the stimulus for the preovulatory LH peak which therefore must be located outside the hypophysiotrophic area of the hypothalamus (51, 52). Further experiments (53, 54) have shown that in the rat, when all the neuronal pathways ending in the hypothalamic area are cut except for a group originating in the POA, both the tonic and cyclic modes of gonadotrophin release remain unaltered. This suggested that the POA contains at least part of the stimulus which triggers LH preovulatory surges and this is fully consonant with the results of brain stimulation reported earlier (1).

Different observations, however, are made in the rhesus monkey (55) where surgical isolation of the medial basal hypothalamus using a modified Halasz method does not impair either tonic or cyclic release of LH and FSH. Thus, in contrast to the rat, the monkey does not seem to require connections from the POA for a gonadotrophin surge and ovulation to occur. These results suggest the importance of the POA for the control of ovulation in the rat. It is known that in this species the preovulatory LH surge is coupled to the diurnal light-dark cycle (1, 16) and the POA is possibly part of the reflex arch which controls the cyclic release of LH (1, 56). In the rhesus monkey, the gonadotrophin surge is independent of any circadian control (57, 58) and thus the connections originating from the POA are probably of minor importance.

- Immunohistochemical methods

Efforts to localize GnRH itself have been attempted either by direct assay of GnRH in isolated portions of the brain or by its identification with immunocytochemical or immunofluorescent techniques. Distribution of GnRH in the brain was first quantified by bioassay (59) and later by immunoassay (60, 61). Within the hypothalamus, the decapeptide is found in greatest amount in the median eminence ; immunocytochemical studies analysed at the ultrastructure level revealed that GnRH stores, appearing in the form of dense vesicles, are associated with 10 to 20 % of the axons composing the external layer (62, 63) ; outside the hypothalamus, concentrations of GnRH have been determined in the POA (61) where the hormone appears to be concentrated in the supra-optic crest (64) and in circumventricular organs (65). Part of the GnRH found in nerve endings of the median eminence is supposed to be synthesized in nerve cell perikarya located in the arcuate nucleus. Nevertheless, immunocytochemistry and immunofluorescence have generally failed to demonstrate GnRH in cell bodies (66, 67, 68) unless the animals have been pretreated in a manner designed to maximise the presence of GnRH e.g. by castration and by colchicine treatment (66). This latter method

suggests that transport of GnRH away from the cell body is normally too rapid to allow its immunohistochemical detection. It is also possible that GnRH acquires its immunological activity only during axonal transport ; (the report by Zimmermann et al, (69) that GnRH can be revealed in nerve cell bodies of the arcuate nucleus in untreated male and female mice is an exception). Recently, GnRH content of the medial basal hypothalamus has been determined by radioimmunoassay after surgical isolation of this region. A marked decrease in GnRH (80-90 %) has been observed in the medial basal hypothalamus of both male and female rats (70), thus suggesting that a large number of GnRH-producing cells are located outside the medial basal hypothalamus but project their axons to that region.

- Steroid implants

Direct effects of sex steroids have been studied at the level of the hypothalamus using implants of crystalline steroid placed at the presumed sites of GnRH production as well as in other suspected steroid-sensitive areas of the brain and plasma gonadotrophins have been investigated. With this method, minute amounts of oestradiol-17 β in quantities demonstrated to produce no systemic effect, were shown to inhibit the post-castration rise in plasma gonadotrophins when placed in the basal hypothalamus, but not when placed in the suprachiasmatic area (71 ; for review, see 72,73). On the other hand, elevation in plasma LH has been found by other authors after median eminence implantation of oestrogen (74). Limitations of the interpretation of median steroid implants have, however, been pointed out by Bogdanove (75) who postulated that the effects of hypothalamic implants are produced by uptake of the implanted steroids into the hypophyseal portal vessels and by transport to the anterior pituitary where the effects are actually exerted.

- Oestrogen receptor sites

Localization of oestrogen effects on GnRH and gonadotrophin release has been studied by following the fate of injected labelled steroids in female animals. Michael's group (76) was the first to use the technique of autoradiography to show a specific oestrogen concentrating effect in certain brain areas of cats. However, not until the development of the powerful and sensitive technique of dry-mount autoradiography (77) was it possible to effectively study in brain and pituitary the precise location - down to a single-cell level - of injected labelled steroids. The findings of Michael (76) have been largely confirmed in many other animal species and there is now agreement that the brain should be considered as an oestrogen target organ : selective uptake of tritiated oestrogen has been reported in the hypothalamus, the POA and the anterior pituitary gland. This important, specific concentration of labelled oestrogen was also observed in the septum, amygdala, circumventricular organs and in the mesencephalon (78,79,80). Concurrent injection of unlabelled oestradiol, oestrone or oestriol decreases ^3H -oestradiol retention in the basal hypothalamus but not in the cortex ; the largest decrease being observed in areas which retain the most ^3H -oestradiol (81). This effect of "cold" hormone pretreatment led to the postulate that specific, high affinity, low capacity, oestrogen binding sites, i.e. receptors, exist in the central nervous system (82). These oestrogen receptors have been identified in the cytosol fraction as well as in the cell nuclei in the oestrogen concentrating areas of the brain and in the anterior pituitary (79,83,84) as described for other oestrogen target organs (85).

Treatment of castrated female rats with exogenous 17β -oestradiol depletes cytoplasmic receptors in uterus, anterior pituitary and hypothalamus with maximal effects observed within one hour (86). The replenishment process which takes place synchronously in all 3 tissues is partly noticeable after 10 h (86). Depletion of cytoplasmic receptor, however, can be correlated with rising levels of nuclear receptor (87, 88). Depletion of cytosol receptor in oestrogen target tissues has been observed on pro-oestrus in adult cycling female rats with replenishment occurring in the morning of oestrus (89). Parallel observations have been made prior to first ovulation either spontaneously occurring (90) or artificially induced with PMSG (91). In the latter case, however, preovulatory depletion in oestrogen cytosol receptors lasts longer (91). The possible implications of this are discussed with the results reported in chapter II and III (for a review on oestrogen/cell interactions, see 85 ; and for a review of this phenomenon in the CNS see 84).

It is tempting to correlate the distribution of oestrogen binding sites in the brain and anterior pituitary gland with oestrogen-sensitive areas of the hypothalamo-pituitary axis and thus to speculate that oestrogen exerts its feedback control on gonadotrophin secretion at two levels i.e. directly at the site of production of the releasing hormone or by modulating the effect of GnRH at the pituitary GnRH receptor after first binding to specific oestrogen receptors. Spona (92) has proposed that the oestrogenic modulation of GnRH action on the anterior pituitary is the result of a sex steroid-GnRH receptor interaction at the pituitary plasma membrane (93). Further, it has now been demonstrated that increases in endogenous oestrogen, e.g. during pro-oestrus, elevate the binding of GnRH to anterior pituitary cells (94). A new approach to the study of this effect of oestrogen is reported in further sections of this thesis.

- Modulation of GnRH response

As early as 1965, Döcke and Dörner (95) recognised that oestrogen exerts a facilitatory effect on gonadotrophin secretion at the level of the pituitary. Already at this time they postulated that this effect could play a role in the cyclic regulation of gonadotrophin secretion, in particular for triggering the preovulatory LH peak. The control exerted by oestrogen on gonadotrophin secretion from the anterior pituitary has since been extensively investigated as synthetic GnRH became available (96, 97, 98).

The responsiveness of the anterior pituitary gland to stimulation with a given dose of GnRH has been found to vary through the reproductive cycle displaying a sharp increase in magnitude at the time of the LH preovulatory peak (93, 99, 100). In adult cycling female rats plasma LH increments observed after injection of a given dose of GnRH are maximum on the day of pro-oestrus with the maximum reached at the time of the LH surge (see for example : 99, 101) ; (Fig. 2). Elevations in plasma FSH after GnRH injection is seen in rats only after administration of the decapeptide through an intravenous infusion (102) or subcutaneous injection (103, 104). In the latter case an increase in FSH response induced by GnRH is observed during the afternoon of pro-oestrus though it is much less dramatic than for LH (101).

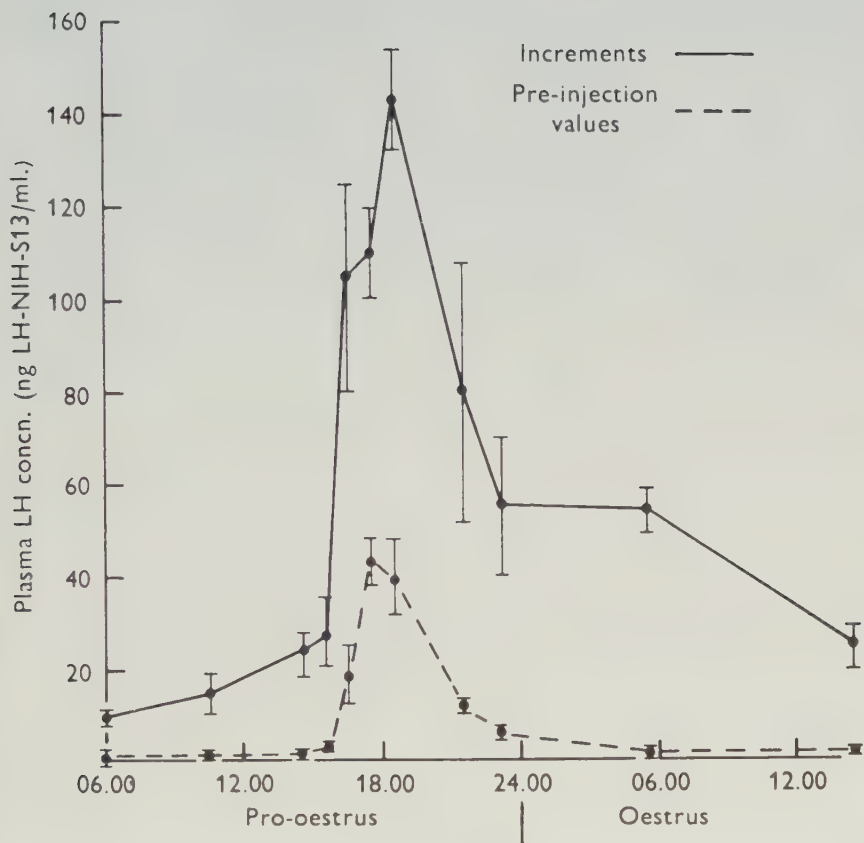


Fig. 2

Profiles of the means $\pm$ SE (four to six animals per group) of the maximal increments (post-injection minus pre-injection values) and pre-injection values of plasma LH in anaesthetized female rats injected with LRF at various times during pro-oestrus and early oestrus.

from Aiyer et al, 1974 (99)

Comparable variations in anterior pituitary sensitivity to GnRH are observed in humans : a progressive increase in responsiveness to GnRH takes place during the course of the follicular phase and reaches a maximum during the mid-cycle gonadotrophin peak (100). Oestrogens have been implicated in this change in pituitary sensitivity to GnRH, observed during the course of the reproductive cycle, as this event roughly parallels the preovulatory elevation in plasma oestrogen (see for example 99). Further, oestrogen pretreatment has been demonstrated to increase the responsiveness of the gland after an initial depressive phase both in rats (see for example 105) and in humans (106,107). But, in addition to this effect of estrogen, GnRH itself has been shown to participate in the midcycle increase in the sensitivity of the gland by a priming effect such that further stimulation induces a larger gonadotrophin response (108). This mechanism may be important for the occurrence of the massive preovulatory LH peak because it may amplify the effects of the endogenous burst in GnRH recently demonstrated in the rat pituitary stalk blood (109). The priming ability of GnRH on the pituitary gland varies through the reproductive cycle and is maximum on pro-oestrus (108) though the importance of this mechanism is not yet established. This problem will be further discussed in the light of the results reported in this thesis (see chapter III).

3. Ontogeny of oestrogen feedback mechanisms.

In the female rat, negative and positive feedback effects exerted by oestrogen on gonadotrophin release develop on a different time scale before reaching a state of functional maturity. We will consider both of these separately.

- Development of negative feedback effect of oestrogen

It has been recognised for some time that ovarian steroids feedback in an inhibitory fashion on gonadotrophin secretion in the prepubertal female rat : both the post-castration rise in gonadotrophin levels and the ability to inhibit this rise with exogenous oestradiol have been found to be operative prior to puberty (2,3,7,110,111,112,113). The quantities of oestradiol required to prevent the post-castration rise in young animals have been found to be smaller than the amount needed in adults, indicating the presence of a more sensitive negative feedback system in immature animals. McCann and Ramirez (110) proposed the use of the term "gonadostat" for the negative feedback control exerted by oestrogen on gonadotrophin secretion. From their own work they concluded that the sensitivity of the gonadostat decreases through the process of puberty, until a new "adult" set point is reached (110,114). According to this theory, as the feedback sensitivity decreases, the level of oestrogen becomes too low to maintain its suppressive effect on gonadotrophin release ; as a consequence, LH and FSH levels rise and ovarian follicular development ensues. This in turn results in an increase in oestrogen production, with induction of a gonadotrophin peak (positive feedback) followed by ovulation. Steele and Weisz (115) have analyzed the negative feedback in castrated rats fitted with intravenous catheters, through which 17β -oestradiol (E2) could be administered in controllable amounts, and sequential daily blood samples could be obtained for LH measurement. With this elegant method the authors demonstrated a peripubertal shift in negative feedback sensitivity to E2 which appeared to be temporarily related to vaginal opening. Data concordant with the "resetting of the gonadostat" hypothesis of the process of puberty have been reported in other species and in particular in man (116), thus demonstrating the general applicability of this principle. The real physiological implication of this theory has been questioned, however, after the observation reported by de Hertogh et al (117) that the metabolic clearance rate of ovarian steroids increases at the time of puberty ; this change could possibly explain an apparent decrease in oestrogen feedback sensitivity. Whether the gonadostat theory adequately describes the mechanisms operative at the onset of puberty is a problem which remains to be resolved.

Although the negative feedback of oestrogen in juvenile animals is recognised as fully operational just before puberty, unsolved new questions have arisen with the recent findings of elevated plasma oestrogen in parallel with elevated plasma FSH in very young female rats, i. e. under 20 days of age (118,

119,120) (see Fig. 3). Questions have been raised on the possible significance of these high plasma FSH levels for ovarian development. Some studies made with antisera against gonadotrophins suggest that high FSH levels are required for the early development of the follicular population (121) whereas others found no effect on follicles when rats received daily injections of antisera to FSH or LH between day 5 and day 15 of life (122,123). The finding of elevated plasma oestrogen together with elevated plasma FSH in early postnatal life of the female rat is hardly compatible with the idea of a fully efficient and operational feedback effect of sex steroids at this early time. However, ovariectomy of 10 day-old female rats results in an elevation of both plasma LH and FSH, when blood was collected 5 days later (124). On the other hand, exogenous oestrogen replacement is able to produce only a limited decrease in the post-castration rise of gonadotrophin in the 15 day-old female rat (125). In comparison to the juvenile condition, it is thus possible that the negative feedback of oestrogen is incompletely developed in the 15 day-old female rat (120). Two current hypotheses are available to explain the poor effectiveness of the negative feedback observed in the infantile condition. The first one is based on the finding by Raynaud et al (126) of an oestrogen binding protein (f-EBP) in the fetal and neonatal rat which binds oestradiol and oestrone but not androgens. In the rat, f-EBP has been identified as alpha-foetoprotein (127,128), the first globulin which appears in mammalian sera during embryonic development. Concentrations of f-EBP are high at birth and decrease progressively during infancy ; f-EBP finally disappears from plasma at about day 21 of life (127,129). Thus, the high plasma oestrogen found at this early time would probably be sequestered by the binding protein and rendered biologically inactive and therefore inefficient in achieving feedback control of gonadotrophin secretion. The other hypothesis states that both cytoplasmic and nuclear binding sites normally found in the adult female hypothalamus, as in other oestrogen target organs (85) have not yet fully matured by day 15 of life. Inability to perform feedback control of gonadotrophin would thus explain the apparent paradox of a coexistence of elevated plasma oestrogen and elevated plasma FSH (118). In their study of the postnatal ontogeny of the oestradiol binding systems in the female rat, Plapinger and McEwen (130,131) have observed two discrete developmental processes. The first of these involved the progressive disappearance, which is complete by day 20 of life, of the foetal type of soluble cytoplasmic oestradiol binding structures present in high concentration throughout the brain of foetal and neonatal rats (130,131). This binding protein has saturable, stereospecific binding properties for 17β -oestradiol but differs in several important aspects from the soluble oestrogen binding protein present in the cytoplasmic fraction of oestrogen target organs of mature rats (129,131,132). In parallel with the progressive disappearance of the foetal oestrogen binding protein, which is homogeneously distributed throughout the brain, the development of the adult-type of cytoplasmic and nuclear binding system takes place in well-defined areas of the brain (130). This development of adult oestrogen receptors is a progressive process as far as cytosol binding capacity is concerned (130). In contrast, the nuclear binding of oestradiol shows rather small modifications during the first 3 weeks of life until an abrupt increase in binding capacity (by a factor of 6) is observed between days 21 and 25 (13). At this age, nuclear oestrogen binding reaches adult levels in the hypothalamus and POA, but stays

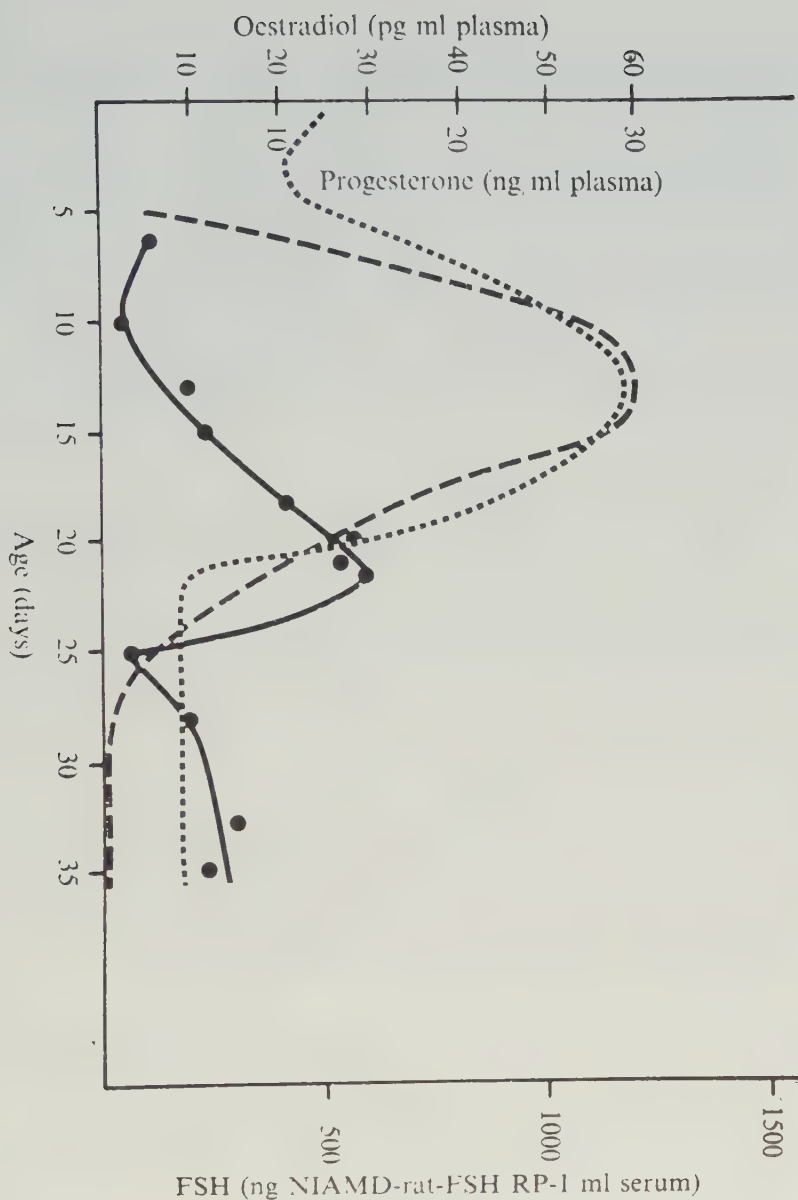


Fig. 3 Plasma progesterone concentrations (•) in immature female rats over the period 5 to 35 days of age, for comparison, the serum FSH (---) and plasma oestradiol (---) concentrations have been included.

From Meijs-Roelofs, 1975 (118)

low in other brain areas (e.g. cortex) (130). Thus, by day 25, the female rat exhibits the striking typical regional specificity in oestrogen binding of the adult (79,133).

At the present level of knowledge, it is hard to incriminate either one of these two factors i.e. the presence of f-EBP in the plasma of infantile animals or the immaturity of their hypothalamic oestrogen binding sites, as the major reason for poor oestrogen feedback control of gonadotrophin secretion. Nevertheless, on balance, the f-EBP is probably the more important factor. Sheridan et al (134) have reported a pattern of brain distribution of oestrogen, analysed through autoradiography, which in 12 day-old female rats, is comparable to that seen in the adult. On the other hand, the elevated f-EBP still does not explain the dissociation between very high plasma FSH values and moderately elevated plasma LH levels in young animals. A satisfactory explanation must await further studies.

- Development of positive feedback

The occurrence of spontaneous puberty has recently been followed with the help of modern hormone assays (90,135,136). Results obtained by these three groups are concordant and show that plasma oestrogen concentrations rise prior to first ovulation. Similarly as prior to cyclic ovulation of the adult female, plasma gonadotrophins (LH and FSH remain low before puberty until the afternoon of the day preceding first ovulation, when a typical LH and FSH preovulatory discharge takes place (90,135,136). As in the adult, pentobarbital is able to block the gonadotrophin surge but is somewhat less efficient at this stage (137). Further, in the PMSG model of precocious puberty (see below) ovulation is suppressed when effects of oestrogen are neutralized by different types of anti-oestrogenic treatments (138,139). Thus, it appears that oestrogen positive feedback on gonadotrophin release needs to be fully operative for the occurrence of first ovulation. The question arises then, as how early puberty can be artificially induced. Many procedures have been used to induce precocious puberty, all resulting in a rise in plasma oestrogen. We shall briefly summarize them with special emphasis on procedures used by the author of this work.

a) Exogenous oestrogen

Since the early work of Hohlweg in 1934 (140), treatment of immature female rats with exogenous oestrogen is known to induce premature sexual maturation (this is the so-called "Hohlweg-Effect"). More recently, in their classical work, Ramirez and Sawyer (141) showed that repeated injections of nearly physiological doses of oestrogen (0.05 µg) starting on day 26 of life resulted in premature "first ovulation".

b) Exogenous gonadotrophins (PMSG)

Gonadotrophins have also been known for a long time to induce precocious sexual maturation. As early as 1929, Kallas (142) reported precocious ovulation in immature female rats coupled in parabiosis with ovariectomised females of the same age. Kallas observed a progressive development of the sexual apparatus of the intact female, leading to ovulation and luteinisation of the developed follicles (142). He interpreted these events as resulting from ovarian stimulation

by pituitary hormones produced by the spayed animal. A single injection of pregnant mare serum (PMSG), which contains mainly FSH, has been shown to induce ovulation in immature female rats with a consistent delay of 3 days (143). As early as 1944, Williams suspected PMSG-induced ovulation to depend on the release of additional endogenous gonadotrophin (144). Today, it is known that a sequence of events comparable to that occurring in the adult female rat i.e. elevation in plasma oestrogen and a peak of LH and FSH, precedes the premature first ovulation induced by PMSG (91). A comprehensive study on the minimal age for induction of precocious puberty has been performed by McCormack and Meyer (145) who reported difficulty in inducing ovulation if PMSG was injected before day 20 of life. Since then, the PMSG model has been extensively used, mainly because it offers the possibility of inducing precocious first ovulation with a short latency (3 days) in a large group of animals together with full synchronisation and high rate of success ($> 95\%$). The second day following the PMSG injection is thus often compared to, and, for convenience reasons, used as a model of the pro-oestrus day of the adult cycle. The PMSG model has also been used in the present work for studying the ontogeny of oestrogen positive feedback on gonadotrophin secretion, and will be referred to in further sections.

c) Brain lesions

A new approach to the study of the onset of puberty was made by Donovan and Van der Werff ten Bosch (146) who originally reported that electrolytic lesions of the anterior hypothalamus can induce precocious sexual maturation of immature female rats, a fact which has first been interpreted as the result of destruction by the lesion, of brain sites mediating negative feedback effects. Ruf et al (147) have further studied the mode of action of brain lesions which induce precocious puberty. In their experiments, direct anodal current (7.5 mC) was passed through a unipolar stainless steel electrode located in the basal hypothalamus of 23 day-old female rats. This type of technique is known to cause a deposition of metallic ions (iron) which provokes an irritation at the periphery of the destroyed tissue (1,148) and is thus probably better qualified by the term "electrochemical stimulation" (148). The effect produced can result in either destruction or stimulation of the tissue in which the electrode has been placed. According to the report by Ruf et al (147), the "lesion-stimulation" placed in the medial basal hypothalamus of 56-65 g, 23 day-old female rats induced ovulation in a large number of rats within 96 or 120 h after placement of the lesion. Increase in oestrogen secretion was demonstrated well before ovulation in these lesioned rats: Ovarian concentration of oestrogen and progestins was already significantly increased 60 min after electrochemical lesion (149); uterine enlargement, which reflects the biological action of the rise in plasma oestrogen, becomes apparent 48 hours after the brain lesion has been performed and further increases until reaching the characteristic ballooned state on the preovulatory day (147). In comparable studies, the levels of plasma gonadotrophins were followed after stimulation of the arcuate nucleus with electrical pulses (150). These authors observed an early rise in plasma LH, taking place during the stimulation itself and reaching a maximum during the first hour after the lesion. An increase in plasma FSH was rather marginal and occurred much later (3-4 h after the electrical brain stimulation) (150). Brain lesion located in the medial basal hypothalamus

have been used by the author, in the present work, to stimulate the endogenous production of oestrogen in immature female rats ; they will be further discussed in the following section of this thesis. Positive feedback of oestrogen on gonadotrophins is also studied in the present work by sequential steroid injections, as earlier discussed (see page 5). In these models, ovulation does not always take place after the induced LH peak.

4. Introduction to the present work

In the course of studies on the mechanism of the onset of puberty we have been interested in the effect exerted by oestrogen on gonadotrophin release mediated through modification of pituitary sensitivity to GnRH stimulation in immature female rats. For the adult animal there is now a large body of evidence that the responsiveness to GnRH varies through the reproductive cycle (99), as well as after pretreatment with oestradiol benzoate (OB). Further, GnRH itself has been demonstrated to increase the pituitary sensitivity by priming the gland so that further stimulation induces a larger response (108). This property of GnRH is maximal on pro-oestrus and is believed to be important for the occurrence of the preovulatory LH peak (108). In studies dealing with other pituitary hormones, Reymond et al (151) have recently reported that responsiveness of the gland to TRH stimulation also varies through the reproductive cycle. It is thus possible that modification of pituitary sensitivity to stimulation with releasing hormones is a mechanism of general importance for the regulation of pituitary hormones secretion.

In the present work, two distinct approaches have been used to study pituitary sensitivity to exogenous synthetic GnRH : In a first series of experiments (Chapter II) we have investigated the effect of short-term (15 h) oestrogen pretreatment on the sensitivity of hemipituitaries to repeated GnRH "pulses" assayed *in vitro* according to a method described in Chapter II. This *in vitro* set up has allowed us to study the ontogeny of the potentiation exerted by oestrogen on the pituitary, which is evident as early as day 5 of life (the earliest time studied). The second approach (Chapter III) has been based on an *in vivo* technique : in these experiments the sensitivity of the pituitary gland was investigated by assaying elevations in plasma LH and FSH in 26 day-old female rats (i.e. roughly 9 days before puberty in our colony). At this age the positive feedback of oestrogen can be made operative, i.e. elevation of plasma oestrogen can result in a rise in gonadotrophins, with either combined exogenous steroid treatment (36) or stimulation of the endogenous production of oestrogens (145,147). We have compared the pituitary responsiveness to one and two GnRH injections applied at 3 different times of day 26, i.e. 1000, 1400 and 1700 h, in female rats in which an LH surge had been induced. These results are compared with the responsiveness found in controls or that observed in animals pretreated overnight with oestrogen, i.e. in animals in which no gonadotrophin surge had been induced. The results will be discussed separately for *in vitro* and *in vivo* works in the respective sections of this thesis.

REFERENCES

1. Everett, J.W. (1964) : Central neural control of reproductive functions of the adenohipophysis.
Physiol.Rev. 44, 373.
2. Moore, C.R. and Price, D. (1932) : Gonadal hormone functions and the reciprocal influence between gonads and hypophysis with its bearing on sex hormone antagonism.
Am. J. Anat. 50, 13.
3. Hohlweg, H. and Dohrn, M. (1932) : Ueber die Beziehungen zwischen Hypophysenvorderlappen und Keimdrüsen.
Klin. Wschr. 11, 233.
4. Greep, R.O. and Chester, J., I. (1950) : Steroid control of pituitary function.
Progr. Hormone Res. 5, 197.
5. Parlow, A.F. (1961) : Bioassay of pituitary luteinizing hormone by depletion of ovarian ascorbic acid.
In : Human Pituitary Gonadotropins ; ed. C.C. Thomas, Springfield, Ill, USA, pp 434.
6. Steelman, S.L. and Pohley, F.M. (1953) : Assay of the follicle stimulating hormone based on the augmentation with human chorionic gonadotropin.
Endocrinology 53, 604.
7. Barraclough, C.A. and Haller, E.W. (1970) : Positive and negative feedback effects of estrogen on pituitary LH synthesis and release in normal and androgen-sterilized female rats.
Endocrinology 86, 542.
8. McPherson III, J.C., Costoff, A. and Mahesh, V.B. (1975) : Influence of estrogen-progesterone combinations on gonadotropin secretion in castrate female rats.
Endocrinology 97, 771.
9. Monroe, S.E., Parlow, A.F. and Midgley, A.R., Jr. (1968) : Radioimmunoassay for rat luteinizing hormone.
Endocrinology 83, 1004.
10. Niswander, G.D., Midgley, A.R., Jr, Monroe, S.E. and Reichert, L.E., Jr. (1968) : Radioimmunoassay for rat luteinizing hormone with antiovine LH serum and ovine LH-¹³¹I.
Proc. Soc. Exptl. Biol. Med. 128, 807.

11. Midgley, A. R. , Jr. (1966) : Radioimmunoassay : A method for human chorionic gonadotropin and human luteinizing hormone.
Endocrinology 79, 10.
12. Midgley, A. R. , Jr. (1967) : Radioimmunoassay for human follicle-stimulating hormone.
J. Clin. Endocrinol. Metab. 27, 295.
13. Yamaji, T. , Dierschke, D.J. , Bhattacharya, A. N. and Knobil, E. (1972) : The negative feedback control by estradiol and progesterone of LH secretion in the ovariectomized rhesus monkey.
Endocrinology 90, 771.
14. Everett, J.W. (1948) : Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat.
Endocrinology 43, 389.
15. Yoshinaga, K. , Hawkins, R.A. and Stocker, J. F. (1969) : Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy.
Endocrinology 85, 103.
16. Schwartz, N.B. (1969) : A model for the regulation of ovulation in the rat.
Recent Prog. Hormone Res. 25, 1.
17. Naftolin, F. , Brown-Grant, K. and Corker, C.S. (1972) : Plasma and pituitary luteinizing hormone and peripheral plasma oestradiol concentrations in the normal oestrous cycle of the rat and after experimental manipulation of the cycle.
J. Endocrinol. 53, 17.
18. Kalra, S. P. and Kalra, P.S. (1974) : Temporal interrelationships among circulating levels of estradiol, progesterone and LH during the rat estrous cycle : Effects of exogenous progesterone.
Endocrinology 95, 1711.
19. Abraham, G. E. , Odell, W.D. , Swerdloff, R.S. and Hopper, K. (1972) : Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17-hydroxy-progesterone, and estradiol-17 β during the menstrual cycle.
J. Clin. Endocrinol. Metab. 34, 312.
20. Kalra, S. P. (1975) : Observation of facilitation of the preovulatory rise of LH by estrogen.
Endocrinology 96, 23.
21. Kahwanago, I. , le Roy Heinrichs, W. , and Hermann, W. L. (1970) : Estradiol "receptors" in hypothalamus and anterior pituitary gland : inhibition of estradiol binding by SH-group blocking agents and clomiphene citrate.
Endocrinology 86, 1319.

22. Shirley, B., Wolinsky, J. and Schwartz, N.B. (1968) : Effects of a single injection of estrogen antagonist on the estrous cycle of the rat. *Endocrinology* 82, 959.
23. Labhsetwar, A.P. (1970) : Role of oestrogen in spontaneous ovulation demonstrated by use of an antagonist of oestrogen ICI : 46,474. *Nature (London)* 225, 80.
24. Labhsetwar, A.P. (1970) : The role of the oestrogens in spontaneous ovulation : Evidence for positive oestrogen feedback in 4 day oestrous cycle. *J. Endocrinol.* 47, 481.
25. Ferin, H., Zimmering, P.E., Lieberman, S. and Vande Wiele, R.L. (1968) : Inactivation of the biological effects of exogenous and endogenous estrogen by antibodies to 17 β -estradiol. *Endocrinology* 83, 565.
26. Ferin, M., Tempone, A., Zimmering, P.E. and Vande Wiele, R.L. (1969) : Effect of antibodies to 17 β -estradiol and progesterone to the estrous cycle of the rat. *Endocrinology* 85, 1070.
27. Yamaji, T., Dierschke, D.J., Hotchkiss, J., Bhattacharya, A.N., Surve, A.H. and Knobil, E. (1971) : Estrogen induction of LH release in the rhesus monkey. *Endocrinology* 89, 1034.
28. Knobil, E. (1974) : On the control of gonadotropin secretion in the rhesus monkey. *Recent Progr. Hormone Res.* 30, 1.
29. Yen, S.S.C., and Tsai, C.C. (1972) : Acute gonadotropin release induced by exogenous estradiol during the mid-follicular phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* 34, 298.
30. Monroe, S.E., Jaffe, R.B. and Midgley, A.R., Jr. (1972) : Regulation of human gonadotropins. XII. Increase in serum gonadotropins in response to estradiol. *J. Clin. Endocrinol. Metab.* 34, 342.
31. Caligaris, L., Astrada, J.J. and Taleisnik, S. (1971) : Biphasic effect of progesterone on the release of gonadotropin in rats. *Endocrinology* 89, 331.
32. Brown-Grant, K. and Naftolin, F. (1972) : Facilitation of luteinizing hormone secretion in the female rat by progesterone. *J. Endocrinol.* 53, 37.
33. Kalra, P.S., Fawcett, C.P., Krulich, L. and McCann, S.M. (1973) : The effects of gonadal steroids on plasma gonadotropins and prolactin in the rat. *Endocrinology* 92, 1256.

34. Beattie, C.W. and Corbin, A. (1975) : The differential effects of diestrous progestagen administration on proestrous gonadotrophin levels.
Endocrinology 97, 885.
35. Caligaris, L., Astrada, J.J. and Taleisnik, S. (1971) : Release of luteinizing hormone induced by estrogen injection into ovariectomized rats.
Endocrinology 88, 810.
36. Caligaris, L., Astrada, J.J., and Taleisnik, S. (1972) : Influence of age on the release of luteinizing hormone induced by oestrogen and progesterone in immature rats.
J. Endocrinol. 55, 97.
37. Ramirez, V.D. and Sawyer, C.H. (1974) : Differential dynamic responses of plasma LH and FSH to ovariectomy and to a single injection of estrogen in the rat.
Endocrinology 94, 987.
38. Brown-Grant, K. (1976) : Control of gonadotropin secretion.
In : Subcellular mechanisms in reproductive neuroendocrinology.
ed. F. Naftolin ; Elsevier Excerpta Medica, Amsterdam.
39. Harris, G.W. and Jacobson, D. (1952) : Function grafts of the anterior pituitary gland.
Proc. Royal Soc. (Series B) 139, 263.
40. Harris, G.W. (1948) : Neutral control of the pituitary gland.
Physiol. Rev. 28, 139.
41. Harris, G.W. (1948) : Electrical stimulation of the hypothalamus and the mechanism of neural control of the adenohipophysis.
J. Physiol. (London) 107, 418.
42. Halász, B., Pupp, L., Unnlarik, S. and Tima, L. (1965) : Further studies on the hormone secretion of the anterior pituitary transplanted into the hypophysiotrophic area of the rat hypothalamus.
Endocrinology 77, 343.
43. Halász, B. (1972) : Hypothalamic mechanisms controlling pituitary function.
Prog. Brain Res. 38, 97.
44. Taleisnik, S. and McCann, S.M. (1961) : Effects of hypothalamic lesions on the secretion and storage of hypophysial luteinizing hormone.
Endocrinology 68, 263.
45. Bishop, W., Krulich, L., Fawcett, C.P. and McCann, S.M. (1971) : The effect of median eminence (ME) lesions on plasma levels of FSH, LH and prolactin in the rat.
Proc. Soc. Exptl. Biol. Med. 137, 1411.
46. Ankomnes -Rodriguez, J. and McCann, S.M. (1967) : The effect of suprachiasmatic lesions on the regulation of LH secretion in the female rat.
Endocrinology 81, 666.

47. Clemens, J.A., Shaar, C.J., Kleber, J.W. and Tandy, W.A. (1971) : Areas of brain stimulatory to LH and FSH secretion. *Endocrinology* 88, 180.
48. Kalra, S.P., Ajika, K., Krulich, L., Fawcett, C.P., Quijada, M. and McCann, S.M. (1971) : Effects of hypothalamic and preoptic electrochemical stimulation on gonadotropin and prolactin release in proestrous rats. *Endocrinology* 88, 1150.
49. Bishop, W., Fawcett, C.P., Krulich, C. and McCann, S.M. (1972) : Acute and chronic effects of hypothalamic lesions on the release of FSH, LH and prolactin in intact and castrated rats. *Endocrinology* 91, 643.
50. Fink, G. and Jamieson, M.G. (1976) : Immunoreactive luteinizing hormone releasing factor in rat pituitary stalk blood : effects of electrical stimulation of the medial preoptic area. *J. Endocrinol.* 68, 71.
51. Halász, B. and Pupp, L. (1965) : Hormone secretion of the anterior pituitary gland after physical interruption of all nervous pathways to the hypophysis-trophic area. *Endocrinology* 77, 553.
52. Halász, B. and Gorski, R. (1967) : Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. *Endocrinology* 80, 608.
53. Tejasen, T. and Everett, J.W. (1967) : Surgical analysis of the preoptico-tuberal pathway controlling ovulatory release of gonadotropins in the rat. *Endocrinology* 81, 1387.
54. Koves, K. and Halász, B. (1970) : Location of the neural structures triggering ovulation in the rat. *Neuroendocrinology* 6, 180.
55. Krey, L.C., Butler, W.R. and Knobil, E. (1975) : Surgical disconnection of the medial basal hypothalamus and pituitary function in the rhesus monkey. I. Gonadotropin secretion. *Endocrinology* 96, 1073.
56. Legan, S.J. and Karsch, F. (1975) : A daily signal for the LH surge in the rat. *Endocrinology* 96, 57.
57. Weick, R.F., Dierschke, D.J., Karsch, F.J., Butler, W.R., Hotchkiss, J. and Knobil, E. (1973) : Periovaratory time courses of circulating gonadotropic and ovarian hormones in the rhesus monkey. *Endocrinology* 93, 1140.

58. Karsch, F.J., Weick, R.F., Butler, W.R., Dierschke, D.J., Krey, L.C. Weiss, G., Hotchkiss, J., Yamaji, T. and Knobil, E. (1973) : Induced LH surges in the rhesus monkey : strength duration characteristics of the estrogen stimulation.
Endocrinology 92, 1740.
59. Crichton, D.B., Schneider, H.P.G. and McCann, S.M. (1970) : Localization of LH-releasing factor in the hypothalamus and neurohypophysis as determined by an in vitro assay.
Endocrinology 87, 323.
60. Palkovits, M., Arimura, A., Brownstein, M., Schally, A.V. and Saavedra, J.M. (1974) : Luteinizing hormone-releasing hormone (LH-RH) content of the hypothalamic nuclei in rat.
Endocrinology 95, 554.
61. Wheaton, J.E., Krulich, L. and McCann, S.M. (1975) : Localization of luteinizing hormone-releasing hormone in the preoptic area and hypothalamus of the rat using radioimmunoassay.
Endocrinology 97, 30.
62. Pelletier, G., Labrie, F., Puviani, R., Arimura, A. and Schally, A.V. (1974) : Immunohistochemical localization of luteinizing hormone-releasing hormone in the rat median eminence.
Endocrinology 95, 314.
63. Sétáló, G., Vigh, S., Schally, A.V., Arimura, A. and Flerko, B. (1975) : LH-RH containing neural elements in the rat hypothalamus.
Endocrinology 96, 135.
64. Wheaton, J.E. Krulich, L. and McCann, S.M.
Endocrinology (in press).
65. Kizer, J.S., Pakovits, M. and Brownstein, M.J. (1976) : Releasing factors in the circumventricular organs of the rat brain.
Endocrinology 98, 311.
66. Barry, J., Dubois, M.P., and Poulain, P. (1973) : LRF producing cells of the mammalian hypothalamus. A fluorescent antibody study.
Z. Zellforsch. 146, 351.
67. Baker, B.L., Dermody, W.C. and Reel, J.R. (1974) : Localization of luteinizing hormone-releasing hormone in the mammalian hypothalamus.
Am. J. Anat. 139, 129.
68. Kordon, C., Kerdhué, B., Pattou, E. and Jutisz, M. (1974) : Immunocytochemical localization of LHRH in axons and nerve terminals of the rat median eminence.
Proc. Soc. Exptl. Biol. Med. 147, 122.
69. Zimmerman, E.A., Hsu, K.C., Ferin, M. and Kozlowski, G.P. (1974) Localization of gonadotropin-releasing hormone (Gn-RH) in the hypothalamus of the mouse by immunoperoxidase technique.
Endocrinology 95, 1.

70. Brownstein, M.J., Arimura, A., Schally, A.V., Palkovits, M. and Kizer, J.S. (1976) : The effect of surgical isolation of the hypothalamus on its luteinizing hormone-releasing hormone content. *Endocrinology* 98, 662.
71. Lisk, R.D. (1960) : Estrogen sensitive centers in the hypothalamus of the rat. *J. Exptl. Zool.* 145, 197.
72. Davidson, J.M. (1969) : Feedback control of gonadotropin secretion. In : *Frontiers in Neuroendocrinology*, Ganong W.F. and Martini L., ed., New York ; pp. 343.
73. McCann, S.M. (1974) : Regulation of secretion of follicle-stimulating hormone and luteinizing hormone. In : *Handbook of Physiology*, section 7, vol. IV, part 2 ; American Physiol. Soc. Washington D.C. ; pp. 489.
74. Palka, Y.S., Ramirez, V.D. and Sawyer, C.H. (1966) : Distribution of biological effects of tritiated estradiol implanted in the hypothalamo-hypophyseal region of female rats. *Endocrinology* 78, 487.
75. Bogdanove, E.M. (1963) : Direct gonad- pituitary feedback ; analysis of the effects of intracranial estrogenic depots on gonadotrophin secretion. *Endocrinology* 73, 696.
76. Michael, R.P. (1965) : Oestrogens in the central nervous system. *Brit. Med. Bull.* 21, 87.
77. Stumpf, W.E. (1968) : Estradiol-concentrating neurons : topography in the hypothalamus by dry-mount autoradiography. *Science* 162, 1001.
78. Stumpf, W.E. (1974) : Anatomical distribution of estrogen in the central nervous system. *Adv. in Biosci.* 15, 77.
79. Stumpf, W.E. (1976) : Localization of receptors in relation to function. In : *Proc. Vth Int. Congress of Endocrinology*, Excerpta Medica, Amsterdam ; in press.
80. Maurer, R.A. and Wooley, D.E. (1975) : ^3H -estradiol distribution in female, androgenised female and male rats at 100 and 200 days of age. *Endocrinology* 96, 755.
81. McEwen, B.S. and Pfaff, D.W. (1970) : Factors influencing sex hormone uptake by rat brain regions. I. Effects of neonatal treatment, hypophysectomy and competing steroid on estradiol uptake. *Brain Research* 21, 1.
82. Kato, J. and Vilee, C.A. (1967) : Factors affecting uptake of estradiol- $6,7\text{-}^3\text{H}$ by the hypophysis and hypothalamus. *Endocrinology* 80, 1133.

83. Eisenfeld, A.J. (1970) : ^3H -estradiol : in vitro binding to macromolecules from the rat hypothalamus, anterior pituitary and uterus. *Endocrinology* 86, 1313.
84. King, R.J.B. and Mainwaring, W.I.P. (1974) : Steroid-cell interactions. Butterworths, London.
85. Zigmond, R.E. (1975) : Steroid effects in the nervous system. In : *Handbook of Psychopharmacology*, Vol. 5, C.L. Iverser, S.D. Iverser and S.H. Snyder, eds. ; Plenum Press, N.Y.
86. Cidlowski, J.A. and Muldoon, T.G. (1974) : Estrogenic regulation of cytoplasmic receptor populations in estrogen-responsive tissues of the rat. *Endocrinology* 95, 1621.
87. Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and de Sombre, E.R. (1968) : A two-step mechanism for the interaction of estradiol with the rat uterus. *Proc. Nat. Acad. Sci. USA*, 59, 632.
88. Anderson, J., Clark, J.H. and Peck, E.J., Jr. (1972) : Oestrogen and nuclear binding sites. *Biochem. J.* 126, 561.
89. Greeley, G.H., Jr., Muldoon, T.G. and Mahesh, V.B. (1975) : Correlative aspects of luteinizing hormone-releasing hormone sensitivity and cytoplasmic estrogen receptor concentration in the anterior pituitary and hypothalamus of the cycling rat. *Biol. Reprod.* 13, 505.
90. Parker, C.R., Jr., and Mahesh, V.B. (1976) : Hormonal events surrounding the natural onset of puberty in female rats. *Biol. Reprod.* 14, 347.
91. Parker, C.R., Jr., Costoff, A., Muldoon, T.G. and Mahesh, V.B. (1976) : Action of pregnant mare serum gonadotropin in the immature female rat : correlative changes in blood steroids, gonadotropins, and cytoplasmic estradiol receptors of the anterior pituitary and hypothalamus. *Endocrinology* 98, 129.
92. Spona, J. (1975) : Sex steroids influence LHRH-receptor interaction. *Endocrinologica Exp.* 9, 167.
93. Schally, A.V., Redding, T.W. and Arimura, A. (1973) : Effect of sex steroids on pituitary responses to LH and FSH releasing hormone in vitro. *Endocrinology* 93, 893.
94. Park, K.R. (1976) : Specific binding of LHRH to the anterior pituitary gland during the oestrous cycle in the rat. *Acta Endocr. (Kbh)* 82, 62.
95. Döcke, F. and Dörner, G. (1965) : The mechanism of the induction of ovulation by oestrogens. *J. Endocrinol.* 33, 491.

96. Schally, A.V., Arimura, A., Kastin, A.J., Matsuo, H., Baba, Y., Redding, R.M., Nair, R.M.G., Debeljuk, L. and White, W.F. (1971) : Gonadotropin-releasing hormone : One polypeptide regulates secretion of LH and FSH. *Science* 173, 1036.
97. Monahan, M., Rivier, J., Burgus, R., Amoss, M., Blackwell, R., Vale, W. and Guillemin, R. (1971) : Synthèse totale par phase solide d'un décapeptide qui stimule la sécrétion des gonadotropines hypophysaires LH et FSH. *C.R. Acad. Sci. (Série D.) Paris* 273, 508.
98. Milhaud, G., Rivaille, P., Garnier, P., Chaussain, J.L., Binet, E. and Job, J.C. (1971) : Synthèse de la LH-RH et effet sélectif sur la libération de l'hormone lutéotrophique chez l'homme adulte. *C.R. Acad. Sci. (Série D.) Paris* 273, 1858.
99. Aiyer, M.S., Fink, G. and Greig, F. (1974) : Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor during the oestrous cycle of the rat. *J. Endocrinol.* 60, 47.
100. Yen, S.S.C., Vanden Berg, G., Rebar, R. and Ehara, Y. (1972) : Variation of pituitary responsiveness to synthetic LRF during different phases of the menstrual cycle. *J. Clin. Endocrinol. Metab.* 35, 931.
101. Zeballos, G. and McCann, S.M. (1975) : Alteration during the estrous cycle in the responsiveness of the pituitary to subcutaneous administration of synthetic LH-releasing hormone (LHRH). *Endocrinology* 96, 1377.
102. Arimura, A., Debeljuk, L. and Schally, A.V. (1972) : Stimulation of FSH release in vivo by prolonged infusion of synthetic LH-RH. *Endocrinology* 91, 529.
103. Greeley, G.H., Jr., Allen, M.B. and Mahesh, V.B. (1974) : FSH and LH response in the rat after intravenous, intracarotid or subcutaneous administration of LH-RH. *Proc. Soc. Exptl. Biol. Med.* 147, 859.
104. Zeballos, G. and McCann, S.M. (1974) : The effect of subcutaneous administration of synthetic luteinizing hormone releasing factor on plasma gonadotropins and prolactin in the rat. *Proc. Soc. Exptl. Biol. Med.* 145, 415.
105. Vilchez-Martinez, J.A., Arimura, A., Debeljuk, L. and Schally, A.V. (1974) : Biphasic effect of estradiol benzoate on the pituitary responsiveness to LH-RH. *Endocrinology* 94, 1300.
106. Keye, W.R. and Jaffe, R.B. (1975) : Strength-duration characteristics of estrogen effects on gonadotropin response to gonadotropin-releasing hormone in women. I. Effects of varying duration of estradiol administration. *J. Clin. Endocrinol. Metab.* 41, 1003.

107. Young, J.R. and Jaffe, R.B. (1976) : Strength-duration characteristics of estrogen effects on gonadotropin response to gonadotropin-releasing hormone in women. II. Effects of varying concentrations of estradiol. *J. Clin. Endocrinol. Metab.* 42, 432.
108. Aiyer, M.S., Chiappa, S.A. and Fink, G. (1974) : A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat. *J. Endocrinol.* 62, 573.
109. Sarkar D.K., Chiappa S.A., Fink G. and Sherwood N.M. (1976) : Gonadotropin-releasing hormone surge in pro-oestrus rat. *Nature* 264, 461.
110. Ramirez, V.D. and McCann, S.M. (1963) : Comparison of the regulation of luteinizing hormone (LH) secretion in immature and adult rats. *Endocrinology* 72, 452.
111. Eldridge, J.C., McPherson III, J.C. and Mahesh, V.B. (1974) : Maturation of negative feedback control of gonadotropin secretion in the female rat. *Endocrinology* 94, 1536.
112. Ojeda, S.R., Kalra, P.S. and McCann, S.M. (1975) : Further studies on the maturation of estrogen negative feedback on gonadotropin release in the female rat. *Neuroendocrinology* 18, 242.
113. McPherson III, J.C., Costoff, A. and Mahesh, V. (1975) : Influence of estrogen-progesterone combinations on gonadotropin secretion in castrated female rats. *Endocrinology* 97, 771.
114. McCann, S.M., Ojeda, S. and Negro-Vilar, A. (1974) : Sex steroid, pituitary and hypothalamic hormones during puberty in experimental animals.
In : *The Control of the Onset of Puberty*, M.M. Grumbach, ed., Wiley and Sons, New York ; pp. 1.
115. Stede, R.E. and Weisz, J. (1974) : Changes in sensitivity of the estradiol-LH feedback system with puberty in the female rat. *Endocrinology* 95, 513.
116. Winter, J.S.D. and Faiman, C. (1972) : Serum gonadotropin concentrations in agonal children and adults. *J. Clin. Endocrinol. Metab.* 35, 561.
117. de Hertogh, R., Ekka, E., Vanderheyden, I. and Hoet, J.J. (1970) : Metabolic clearance rates and the interconversion factors of estrone and estradiol-17 β in the immature and adult female rat. *Endocrinology* 87, 874.

118. Meijs-Roelofs, H.M.A., de Greef, W.J. and Uilenbroek, J.Th.J. (1975) : Plasma progesterone and its relationship to serum gonadotrophins in immature female rats.
J. Endocrinol. 64, 329.
119. Döhler, K.D. and Wuttke, W. (1975) : Changes with age in levels of serum gonadotrophins, prolactin, and gonadal steroids in prepubertal male and female rats.
Endocrinology 97, 898.
120. Ojeda, S.R. (1976) : Maturation of the control of gonadotropin and prolactin release in the rat.
Ann. Biol. anim., Biochim. Biophys. 16, 329.
121. Eshkol, A. and Lunenfeld, B. (1972) : Gonadotropic regulation of ovarian development in mice during infancy.
In : Gonadotrophins ; Saxena B.B., eds., Wiley and Sons, New York ; pp. 335.
122. Schwartz, N.B., Aderson, C.H., Nequin, L.G. and Ely, C.A. (1974) : Follicular maturation.
in : Control of the Onset of Puberty ; MM. Grumbach, ed., Wiley and Sons, New York ; pp. 367.
123. Uilenbroek, J.Th.J., Arendsen de Wolf-Exalto, Els, and Welschen, R. (1967) : Studies on the significance of the high levels of follicle-stimulating hormone for follicular development in immature rats.
Ann. Biol. anim., Biochim. Biophys. 16, 297.
124. Döhler, K.D., Von zur Mühlen, A. and Döhler, U. (1976) : Estrogen-gonadotropin interaction in postnatal female rats and the induction of anovulatory sterility by treatment with an estrogen antagonist.
Ann. Biol. anim., Biochim. Biophys. 16, 363.
125. Ojeda, S.R. and Ramirez, V.D. (1973/74) : Short-term steroid treatment effect on plasma LH and FSH in castrated rats from birth to puberty.
Neuroendocrinology 13, 100.
126. Raynaud, J.P., Mercier-Bodar, C. and Baulieu, E.E. (1971) : Rats estradiol binding plasma protein (EBP).
Steroids 18, 767.
127. Nunes, E., Engelmann, F., Benassayang, C. and Jayle, M.F. (1971) : Identification et purification préliminaire de la foeto-protéine liant les oestrogènes dans le serum de rats nouveau-nés.
C.R. Acad. Sci. (Série D) 273, 831.
128. Uriel, J., Bouillon, D. and Dupiers, M. (1975) : Affinity chromatography of human, rat and mouse alpha-fetoprotein on estradiol sepharose adsorbents.
FEBS Letters 53, 305.

129. Uriel, J., Bouillon, D., Aussel, C. and Dupiers, M. (1976) : Alpha-fetoprotein : the major high-affinity estrogen binder in rat uterine cytosols.
Proc. Nat. Acad. Sci. USA 73, 1452.
130. Plapinger, L. and McEwen, B.S. (1973) : Ontogeny of estradiol-binding sites in rat brain. I. Appearance of presumptive adult receptors in cytosol and nuclei.
Endocrinology 93, 1119.
131. Plapinger, L., McEwen, B.S., and Clemens, L.E. (1973) : Ontogeny of estradiol binding sites in rat brain. II. Characteristics of a neonatal binding macromolecule.
Endocrinology 93, 1129.
132. McEwen, B.S., Lieberburg, I., MacLusky, N. and Plapinger, L. (1976) : Interaction of testosterone and estradiol with the neonatal rat brain : protective mechanism and possible relationship to sexual differentiation.
Ann. Biol. anim., Biochim. Biophys. 16, 471.
133. Zigmond, R.E. and McEwen, B.S. (1970) : Selective retention of oestradiol by cell nuclei in specific brain regions of the ovariectomized rat.
J. Neurochem. 17, 889.
134. Sheridan, P.J., Sar, M. and Stumpf, W.E. (1974) : Autoradiographic localization of ³H-estradiol or its metabolites in the central nervous system of the developing rat.
Endocrinology 94, 1386.
135. Meijs-Roelofs, H.M.A., Uilenbroek, J.Th.J., de Greef, W.J., de Jong, F.H. and Kramer, P. (1975) : Gonadotrophins and steroid levels around the time of first ovulation in the rat.
J. Endocrinol. 67, 275
136. Ojeda, S.R., Wheaton, J.E., Jameson, H.E. and McCann, S.M. (1976) : The onset of puberty in the female rat : changes in plasma prolactin, gonadotrophins, luteinizing hormone-releasing hormone (LH-RH) and hypothalamic LH-RH content.
Endocrinology 98, 630.
137. Osman, P. and Meijs-Roelofs, H.M.A. (1976) : Effects of sodium pentobarbitone administration on gonadotropin release, first ovulation and ovarian morphology in pubertal rats.
J. Endocrinol. 68, 431.
138. Cappola, J.A. and Perrine, J.W. (1965) : Influence of two nonsteroidal antiestrogens on vaginal opening and PMS- induced ovulation in rats.
Endocrinology 76, 865.
139. Ferin, M. Zimmering, P.E. and Vande Wiele, R.L. (1969) : Effect of antibodies to estradiol-17 β on PMS-induced ovulation in immature rats.
Endocrinology 84, 893.

140. Hohlweg, W. (1934) : Veränderungen des Hypophysenvorderlappens und des Ovariums nach Behandlung mit grossen Dosen von Follikelhormon. *Klin. Wsch.* 13, 92.
141. Ramirez, V.D. and Sawyer, C.H. (1965) : Advancement of puberty in the female rat by estrogen. *Endocrinology* 76, 1158.
142. Kallas, H. (1929) : Puberté précoce par parabiose. *C.R. Soc. Biol. Paris* 100, 979.
143. Cole, H.H. (1937) : Superfecundity in rats treated with mare gonadotropic hormone. *Am. J. Physiol.* 119, 704.
144. Williams, P.C. (1944/46) : Studies of the biological action of serum gonadotrophin. 3. Role of endogenous gonadotrophin. *J. Endocrinol.* 4, 137.
145. McCormack, C.E. and Meyer, R.K. (1964) : Minimal age for induction of ovulation with progesterone in rats : evidence for neural control. *Endocrinology* 74, 793.
146. Donovan, B.T. and Van der Werff ten Bosch, J.J. (1956) : Precocious puberty in rats with hypothalamic lesions. *Nature (London)* 178, 745.
147. Ruf, K.B., YoungLai, E.V. and Holmes, M.J. (1974) : Induction of precocious sexual maturation in female rats by electrochemical stimulation of the brain. *Brain Research* 78, 437.
148. Velasco, H.E. (1972) : Opposite effects of platinum and stainless steel lesions of the amygdala on gonadotropin secretion. *Neuroendocrinology* 10, 301.
149. YoungLai, E.V. Holmes, M.J. and Ruf, K.B. (1976) : Changes in concentration of LH, FSH and estrogen in the immature female rat during precocious sexual maturation induced by electrochemical stimulation of the brain. *Hormone Res.*, in press.
150. Moll, J., Meijs-Roelofs, H.M.A., Kramer, P. and Dullaart, J. (1976) : Effects of electrical stimulation and of direct current and high frequency lesions of the hypothalamus on gonadotrophin release and puberty in female rats. *Ann. Biol. anim., Biochim. Biophys.* 16, 433.
151. Reymond, M. and Lemarchand-Béraud, Th. (1976) : Effects of oestrogens on prolactin and Thyrotrophin responses to TRH in women during the menstrual cycle and under oral contraceptive treatment. *Clinical Endocrinology*, 5, 429.
152. Drouin, J. and Labrie, F. (1976) : Selective effect of androgens on LH and FSH release in the anterior pituitary cells in culture. *Endocrinology* 98, 1528.

CHAPTER II

IN VITRO STUDIES OF THE EFFECTS OF OESTROGEN
PRETREATMENT ON THE SENSITIVITY OF IMMATURE
FEMALE RAT PITUITARY TO STIMULATION WITH GONA-
DOTROPHIN RELEASING HORMONE

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I. SUMMARY

The effects of oestrogen priming on the sensitivity of the anterior pituitary to stimulation with GnRH was investigated in immature female rats using a new organ culture technique. Hemipituitaries obtained from animals primed with a single dose of oestradiol benzoate (OB ; 20 µg/100 g body weight) released significantly more LH when pulsed with GnRH (4×10^{-9} mol/l) than did control hemipituitaries. This potentiating effect was detectable as early as 5 days after birth. The response in terms of FSH release was variable and much smaller. However, after the application of a second pulse of GnRH, bigger increases of FSH secretion were obtained after OB treatment. This was most striking in animals approaching maturity (25 and 30 days of age). LH secretion remained high compared with controls after a second stimulation. These results were compared with those obtained from animals primed in such a way as to produce elevated levels of endogenous oestrogen on day 26 of life. Thus, hemipituitaries were obtained from animals which had received either two injections of OB, an injection of pregnant mare serum gonadotrophin (PMSG) or a unilateral brain lesion placed in the basal hypothalamus.

Pituitary tissue was stimulated as before with a pulse of GnRH. Two injections of OB resulted in a large increase in sensitivity to stimulation. On the other hand, both PMSG and lesion treatment severely reduced the sensitivity such that the release of LH and FSH was significantly inhibited.

These results show that the immature female rat pituitary responds to oestrogen treatment in a manner similar to the adult situation and considerably before the time at which a positive feedback response of oestrogen can be elicited. In contrast, though PMSG and lesion-treated animals have been used as models with which to study ovulation, it appears that pituitary sensitivity to GnRH stimulation is quite different to that observed in the adult cycling animal.

II. INTRODUCTION

The facility with which gonadotrophin releasing hormone (GnRH) is able to stimulate secretion of gonadotrophins from the anterior pituitary gland is greatly modified by oestradiol. The effect is normally biphasic, such that an initial suppression of stimulation is followed by an increase in responsiveness (Cooper, Fawcett and McCann, 1974 ; Vilchez-Martinez, Arimura, Debeljuk and Schally, 1974). The pronounced augmentation of sensitivity observed on the day of pro-oestrous in the adult cycling rat has been well described and correlates with the endogenous elevation in oestrogen levels occurring at this time (see for example Aiyer, Fink and Greig, 1974 ; Ferland, Borgeat, Labrie, Bernard, De Lean and Raynaud, 1975). The phenomenon of increased sensitivity has been investigated in ovariectomised, oestrogen-primed animals both in vivo (Aiyer, Sood and Brown-Grant, 1976) and in vitro (Hobson and Hansel, 1974 ; Apfelbaum and Taleisnik, 1976). Thus, within 24 hours after a subcutaneous injection of oestrogen, the response of the pituitary to stimulation in vitro with GnRH was greatly increased.

Our interest in the role of oestrogen in the mechanisms controlling the onset of puberty in the rat, and its involvement in precocious sexual maturation as a result of hypothalamic lesions (Ruf, Kitchen and Wilkinson, 1976 ; de Ziegler, Wilkinson and Ruf, 1976) led us to study the potentiating effect of oestrogen on the pituitary response to GnRH. We tested in vitro the sensitivity to stimulation of hemipituitaries obtained from immature female rats primed in various ways to increase circulating levels of oestrogen. The ontogeny of the effect was studied in animals which had received a single injection of oestradiol benzoate (OB). In addition, three other types of treatment were used to increase oestrogen levels ; all three manipulations are known to induce a gonadotrophin surge in the immature female rat (a) a double injection of OB (Caligaris, Astrada and Taleisnik, 1972); (b) treatment with pregnant mare serum gonadotrophin (PMSG) (Wilson, Horth, Endersby and McDonald, 1974) ; and (c) unilateral electrochemical stimulation of the basal hypothalamus (Ruf, YoungLai and Holmes, 1974 ; YoungLai, Holmes and Ruf, 1976).

III. MATERIALS AND METHODS

Female rats (Sprague-Dawley ; SIV-50) were bred on the premises (lights on 0700 to 1900 h) and obtained as litters of 6 on days 5, 11, 21, 23, 24 and 30 of life. The age at which vaginal opening was normally observed, approximating the onset of puberty, was about 35 days after birth. The rats used in the lesion experiments were of the same strain but were obtained from the Institute of Veterinary Medicine, University of Zurich, on day 21 of life ; these rats, unlike the ones above, represented a selection of approximately the top 10 % of the weight distribution found in this population. Only those animals reaching a weight of > 56 g on day 23 of life were used.

Preparation of animals

1. Ontogeny experiments.

Groups of six animals from each of the age categories 5, 11, 21, 24 and 30 days, were injected at 17.30 h with peanut oil (0.1 ml s.c.) or OB in oil (20 μ g per 100 g body weight). The rats were killed by decapitation the following morning at 10.00 h and the pituitaries placed in organ culture (see below).

2. Double injection of OB, according to the method of Caligaris et al, 1972 :

Groups of six rats, 23 days of age, were injected with peanut oil (0.1 ml s.c.) or 10 μ g OB in oil at noon. These injections were repeated at the same time on day 25. At 10.00 h on day 26, the animals were killed by decapitation and their pituitaries placed in culture. Uteri were also dissected and quickly weighed.

3. Treatment with PMSG :

Groups of six rats, 24 days of age, were injected with saline or with PMSG (10 i.u. ; 10.00 h). The animals were killed at 10.00 h on the morning of day 26 and their pituitaries placed in culture.

4. Animals subjected to a unilateral electrochemical stimulation of the basal hypothalamus (Ruf et al, 1974) :

Briefly this consisted of the passage of a direct anodal current 0.5 mA, 15 secs) through a stainless steel electrode whose uninsulated tip was stereotactically placed near the origin of the pituitary stalk. The animals used were 23 days of age and weighed > 55 g. Groups of 5 animals, together with controls (i.e. both untouched animals and sham-stimulated animals) were killed at 60 mins. and 72 h after stimulation (i.e. 10.00 h, day 26). Pituitaries were dissected out and immediately placed in culture.

Culture technique

Anterior pituitaries were dissected free of the posterior lobe and placed in a drop of 1 % bovine serum albumin (BSA ; Fraction V, SIGMA) in phosphate

buffered saline (PBS ; pH 7.2) on a microscope slide. Under a dissection microscope the pituitary was carefully cut in half (except those from 5 day old rats which were used whole ; this was to prevent inflicting undue damage on these very small glands). Each half was placed on a separate stainless steel grid in an organ culture dish (Falcon No. 3010) containing 0.5 ml of modified Medium 199 (GIBCO, catalogue No. BCL 193). The standard medium was supplemented with sodium pyruvate (1 mmol/l), MEM non-essential amino acids (GIBCO ; 1 ml of 100 x conc. solution in 100 ml final medium), glucose (500 mg/100 ml), fungizone (1.25 µg/ml) and HEPES buffer (20 mmol/l). The medium was distributed to the dishes after it was first gassed at 37° C with 95 % O₂/CO₂. The cultures, with the pituitary tissue lying at the gas/medium interface, were incubated at 37° in a water-saturated atmosphere of 95 % air/CO₂. The cultures were pre-incubated for two separate 60 min. periods, after which the medium was discarded and replaced with further medium (37° ; gassed). This was to allow the initial high levels of hormone secretion, into the medium, to stabilise. The GnRH stimulus (4.2. x 10⁻⁹ mol/l ; 5 ng/ml) was applied as a "pulse" i.e. the pituitary pieces were incubated for 15 min. in the presence of GnRH before the medium was discarded and replaced with fresh medium lacking GnRH. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) released into the medium during a further 60 min. incubation period were assayed using NIH kits, and the results expressed in terms of NIAMDD RP-1 standards. In some experiments, at the end of this 60 min. incubation period, a second pulse of GnRH was applied, and the medium was collected from a further 60 min. incubation in the absence of GnRH. The incubation medium was diluted with 1 % BSA in PBS and stored at -30° C until assayed for LH and FSH.

Experiments were conducted to determine the level of basal secretion, in the absence of GnRH, over a period of four hours (4 x 60 min. incubations). In addition, for one age group (24 days) a comparison was made between the quantity of LH and FSH released after pulsatile stimulation, with that released after the GnRH was allowed to remain in the incubation medium for both of the 60 min. periods of stimulation.

In an attempt to compare the effects of OB *in vivo* with a possible influence *in vitro*, we have incubated pituitary halves from untreated 24 day old female rats with OB (10⁻⁹ mol/l) for a period of 16 h. (18.00 h to 10.00 h) which corresponds to the injection/sacrifice time in the other experiments. The incubation medium was identical to that used previously except for the addition of 5 % (v/v) foetal calf serum (FCS). The OB was added as a solution in propane diol (final concentration, 0.2 % v/v). Propane diol was added to control cultures at the same concentration. After the overnight incubation the medium was discarded and replaced with medium lacking FCS. Incubation was continued for 60 min, the medium was collected and stored, and then the pituitary halves were stimulated with two consecutive pulses of GnRH, as described above.

Synthetic GnRH was from Beckman. Statistical analysis was by unpaired Student's t-test ; *** p < 0.001 ; ** p < 0.005 ; * p < 0.01 ; ° p < 0.05 ; NS = not significant.

IV. RESULTS

Pituitary and uterine weights.

Single injection of OB :

In the ontogenetic studies in which a single injection of OB was used only pituitary weights were recorded. These varied from a mean of 0.5 mg (day 6), 1.2 mg (day 12), 2.4 mg (day 22) to 3.3 mg (day 25). No significant differences in weight were observed between pituitaries from OB-treated animals and those from controls in these age groups. In day 31 animals, however, OB-treated pituitaries were heavier (3.9 mg compared to 3.4 mg, $p < 0.01$).

Double injection of OB :

Pituitary weights for treated and control groups were 3.55 ± 0.14 mg and 2.86 ± 0.14 mg respectively ($p < 0.01$). The corresponding uterine weights were 131 ± 10 mg and 35.8 ± 2.7 mg ($p < 0.001$).

PMSG treatment :

Pituitary weights for treated and control animals were 3.6 ± 0.16 mg and 2.8 ± 0.07 mg respectively ($p < 0.01$). The corresponding uterine weights were 148 ± 9 mg and 43.2 ± 3 mg ($p < 0.001$).

Brain lesions :

Pituitary weights remained the same for treated and control groups until day 26 i.e. 3 days after placement of the brain stimulus ; at this time, the corresponding weights were 3.36 ± 0.12 and 2.90 ± 0.08 mg ($p < 0.01$). Uterine weights slowly increased from day 23 through to day 26 as previously reported (Ruf et al, 1974), indicating elevated levels of circulating oestrogen.

In all experiments in which a difference in pituitary weight was observed, between treated and control groups, secretion of LH and FSH is expressed as ng/mg of tissue/60 min.

Basal secretion

Experiments were conducted to determine whether the different oestrogen priming regimes had any substantial effect on the basal secretion of LH and FSH i.e. without GnRH stimulation. This was studied by collecting four consecutive 60 min. incubation media, beginning with the first incubation after placing the pituitary in culture. No large differences in basal secretion were observed between treated and control cultures. Fig. 1 illustrates the pattern of secretion of LH observed in two age groups. It can be seen that 120 mins in culture is sufficient time to stabilise basal output, and thus is the point at which the GnRH stimulus is applied. Fig. 2 illustrates the basal secretion observed

from pituitaries obtained from animals receiving two injections of OB, and their controls. The large differences in secretion rate (top of Fig. 2 ; ng/ml) became identical when corrected for tissue weight (bottom of Fig. 2 ; ng/mg). In all experiments involving GnRH stimulations, the second preincubation i.e. 60 to 120 min. was collected and assayed. No large differences in secretion rates were observed either for LH or FSH during this period. The results of the secretion experiments described below have been expressed in terms of ng of gonadotrophin per ml of medium except in those cases where pituitary weights differed between experimental groups. In these experimental results, secretion is in terms of ng per mg of tissue.

Ontogeny of the effect of a single injection of OB on pituitary sensitivity to GnRH.

Fig. 3 illustrates the effects on LH and FSH secretion of a single pulse of GnRH delivered to pituitary halves removed from rats either treated with OB (20 µg/100 g body weight) or oil. The results are expressed as the percentage increase in stimulated secretion after OB treatment compared with the stimulated secretion from controls. At all ages tested, a large, highly significant increase in LH release is observed after OB, indicating that the potentiating effect of OB on pituitary sensitivity in the rat is present from a very early age. In terms of FSH release the response was much less and rather variable from one age group to another. As noted earlier, pituitary weights in the 31 day group differ significantly. However, if these figures are converted to ng of LH and FSH released per mg of tissue, differences in response remain highly significant : 2309 ± 185 (12) (OB ; LH) and 1138 ± 98 (10) (control ; LH ; $p < 0.001$) ; and for FSH the respective figures are : 523 ± 46 (12) and 374 ± 29 (11) ng/mg ($p < 0.02$).

Fig. 4 shows the results of an identical stimulus applied 60 min. after the first and using the same pituitary halves as in Fig. 3. Except for day 22, the response in terms of LH remains at a high level, compared with controls. In addition we can now observe a large increase in FSH release on days 25 and 31. An inspection of the control stimulation values (i.e. stimulation of untreated hemipituitaries with GnRH) reveals that no such increase is observed after the second stimulation.

Comparison of "pulse" stimulation with continuous presence of GnRH

The use of a pulse stimulation with GnRH in this culture system was adopted in an attempt to simulate the effects of an intravenous injection of GnRH given to the intact animal. The half life of injected GnRH is of the order of seven minutes (Redding and Schally, 1973 ; Ben-Jonathan, Mical and Porter, 1974), whilst 80 % - maximal binding of labelled GnRH to pituitary plasma membranes has been estimated at 10 mins. (Spona, 1975a). We have chosen a pulse time of 15 mins. taking these figures into account as well as the practical difficulties of changing culture media in large numbers of dishes. The 25 day old preparation was used to determine whether a difference in response exists between a pulse and the continuous presence of GnRH. Table 1 indicates that for LH release, at this age, certain differences in response are apparent. Hemipituitaries from OB treated animals respond identically to either stimulus. On the other hand, control cultures respond in a significantly different fashion to the two stimuli. The pulse of GnRH releases much less LH, which effectively increases the difference in response between treated and control cultures. However, even with a 60 min.

incubation with GnRH, the treated pituitaries still retain their sensitised state with respect to LH release ($p < 0.005$). Once again, the response to stimulation of FSH release is extremely variable.

Effects on pituitary sensitivity of three treatments known to produce an LH surge in the immature rat.

As described under Materials and Methods, we have compared the effects of 1) a double injection of OB ; 2) an injection of PMSG and 3) a hypothalamic lesion, on the sensitivity of hemipituitaries to a single pulse of GnRH. The animals were sacrificed at 10.00 h on day 26 of life. Fig. 5 illustrates that, as before, treatment with OB (designated OB/OB) sensitises the pituitary to stimulation with GnRH. Both LH and FSH release were significantly elevated over control values. Surprisingly, both PMSG and lesion treatment resulted in a highly significant inhibition of release of both hormones compared with controls.

Acute effects of hypothalamic lesion on pituitary sensitivity to GnRH.

The mode of action of brain lesions in the induction of precocious sexual maturation remains unclear. One possibility is that the site of the lesion, rich in endogenous GnRH, releases of gonadotrophins from the anterior pituitary (Ruf, Wilkinson and de Ziegler, 1975). It seemed of interest, therefore, to test the sensitivity of the pituitaries from lesioned animals, removed 60 min. after placement of the lesion. Pituitaries were removed and halved from lesioned, sham-lesioned and control (untouched) animals. After the usual 2 x 60 min. pre-incubation period, the halves were stimulated with two consecutive pulses of GnRH, as described above. No differences were observed between any of the groups in the basal release or stimulated release of LH (results not shown). However, FSH secretion (Fig. 6) appeared to be inhibited in pituitary tissue from both lesioned and sham-lesioned animals, such that a reduced response was observed after the second stimulation.

Effects of incubation with OB during 16 h.

We have attempted to reproduce *in vitro* a possible direct effect of OB on pituitary tissue removed from 24 day old female rats. We estimated that the plasma concentration of estrogen following a single injection of OB (20 $\mu\text{g}/100 \text{ g}$ body weight) should be a maximum of 10^{-9} mol/l . (Legan, Coon and Karsch, 1975). Thus, hemipituitaries were incubated overnight with and without OB at this concentration. After 16 h the medium was discarded and a single 60 min. preincubation carried out in the absence of further OB. The halves were then stimulated twice, as described above. Fig. 7 shows that basal secretion was identical to the baseline established in previous experiments (cf Fig. 1). This indicated that extensive cellular necrosis and discharge of hormone was probably not taking place. Further confirmation of this point is seen (Fig. 7) in the results from the first stimulation. OB-treated and control tissue have a similar response, which itself is indistinguishable from that obtained with tissue of the same age which had not been incubated overnight (Fig. 3 ; control value, day 25). Furthermore, Fig. 7 also shows that a significant increase in LH secretion is observed from the OB treated tissue when stimulated a second time.

V. DISCUSSION

The availability of synthetic GnRH has allowed extensive studies to be performed on the biochemistry, physiology and clinical aspects of its interaction with the anterior pituitary in man and a variety of experimental animals. A number of these studies were made possible by directly investigating the effects of GnRH on the secretory response of pituitary tissue maintained *in vitro*. This aspect alone is extensively documented and includes work on hemipituitaries in organ culture (Schally, Redding and Arimura, 1973; Schafer and McShan, 1974; Apfelbaum and Taleisnik, 1976; Martin and Klein, 1976) superfusion studies (Tedward and Gilbertson, 1976; Kilpatrick, Collins and Newton, 1976; Dowd, Barofsky, Chaudhuri, Lloyd and Weisz, 1975; Osland, Gallo and Williams, 1975) and the establishment of monolayer cultures of pituitary tissue (Vale, Grant, Amoss, Blackwell and Guillemin, 1972; Steinberger and Chowdhury, 1974; Tang and Spies, 1975; Drouin and Labrie, 1976).

In the course of our studies on the sexual maturation of the female rat it became important to investigate the possibility that the sensitivity of the anterior pituitary toward stimulation with GnRH might change according to age and/or hormonal environment. We have chosen to look at these effects *in vitro* largely because in this way it is possible to study the pituitary in isolation, free of neural influences, for example. Also, by using hemipituitaries, experimental numbers can be increased, animal costs reduced and statistical analysis rendered more precise. The particular problem that we have investigated - the influence of oestrogen priming in immature animals - precluded the use of monolayer cultures, since possibly any effects of previous steroid exposure of pituitary cells, *in vivo*, would be lost after enzyme treatment during the process of tissue dispersal. On the other hand, the powerful technique of superfusion, with all its obvious advantages, could not be used because of its sophistication. The rather specialised equipment needed for this approach effectively prevented the use of relatively large numbers of hemipituitaries obtained from both treated and control groups of experimental animals. We have therefore attempted to improve on the well-tried techniques of pituitary organ culture by using individual culture dishes which allow hemipituitaries to rest on a steel grid placed at the gas-medium interface. This is essentially the method used successfully by Klein and Weller (1970) in their studies on pineal, and which enables these glands to remain viable for approximately six days. Independently of our own work, Martin and Klein (1976) have recently extended the pineal methodology to include pituitary tissue (from 5 day old rats) and report the absence of cellular necrosis after a culture period of 4 days. In addition to the use of this *in vitro* system we have incorporated one of the advantages of the superfusion method, namely that of "pulsing" the tissue with GnRH. The reasons for this have been outlined in the Results section.

The data obtained from the experiments which involved a single injection of OB followed, 16 h later, by pulsatile stimulation *in vitro* clearly demonstrate that OB is able to sensitise the pituitary of immature female rats to stimulation with GnRH. This observation extends the work already described in the adult (Hobson and Hansel, 1974 ; Apfelbaum and Taleisnik, 1976) and shows that the effect is detectable shortly after birth. The overall lack of responsiveness in terms of FSH release is modified at the second stimulation such that pituitaries from treated animals of 25 and 31 days of age release large amounts of FSH compared with controls. This delayed response in FSH secretion is also observable in the adult rat after stimulation with GnRH (Zeballos and McCann, 1975) or during the oestrous cycle (de la Cruz, Coy, Vilchez-Martinez, Arimura and Schally, 1976). Our results suggest that oestrogens may play some part in the genesis of this effect, either in the continued presence of GnRH or as the result of pulsatile stimulation.

Though the dose of OB used is grossly pharmacological it is of interest that only a positive (i.e. sensitisation) response is observed. Caligaris et al (1972) have shown that a positive feedback effect of OB (i.e. to provoke the release of gonadotrophins, *in vivo*) is not observable with a single injection of OB before 28 days of age in the rat. Moreover, Barraclough and Turgeon (1975) primed 5 day old female rats with OB and tested pituitary sensitivity 24 h later with a single injection of GnRH. In this case a significant inhibition of the response, in terms of LH release, was reported. Thus the anterior pituitary of these young rats, despite the demonstrated sensitised state which we have described here, are unable to respond to GnRH in the intact animal.

The mechanism of sensitisation of pituitary tissue by OB is not known, though the work of Spona (1974, 1975b) and others (Park, Saxena and Gandy, 1976) implicate an increase in binding of GnRH to (presumably) gonadotroph plasma membranes, brought about by oestrogen. Further, Park et al (1976) have demonstrated that the increase in sensitivity of the anterior pituitary which takes place during pro-oestrous, to stimulation with GnRH, is paralleled not only by a rise in oestrogen levels but by an increased binding of GnRH as well. Despite the fact that the method by which oestrogen increases GnRH binding is obscure, it is possible to suggest that a similar mechanism is operative in the immature rats of the present study. Specific oestrogen binding proteins are known to be present in 5 day old female rat pituitary (MacLusky, Chaptal and McEwen, 1975) and the presence of GnRH receptors is implied from the results of Schafer and McShan (1974) who demonstrated that even fetal rat pituitary responded to GnRH, *in vitro*, by the release of LH and FSH. That oestrogen can exert a direct effect on the pituitary, in the absence of endogenous GnRH, is suggested by the results of the experiment (Fig. 7) in which incubation overnight in the presence of OB, was carried out. These preliminary results show a clear potentiation of response after the second pulse of GnRH. Of importance also was the lack of an inhibitory effect of OB, reported by others using higher concentrations of OB (Schally et al, 1973 ; Tang and Spies, 1975). Further work is necessary to optimise OB concentrations, and it is interesting to note that an unspecified concentration of oestradiol is able to sensitise monolayer cultures of anterior pituitary to stimulation with GnRH (Drouin, Veilleux, Normand and Labrie, 1975).

The results (Fig. 5) of the experiments in which we have used three methods of inducing LH surges in the immature female rat are somewhat difficult to account for. Just as for a single priming injection of OB, a double injection is able to induce a sensitised pituitary response to GnRH though the effect is a little less than for a single injection of OB. In complete contrast, in animals of the same age sacrificed at the same time, a significant inhibition in response to GnRH was observed after either PMSG treatment or brain stimulation. In the case of PMSG induced ovulation the results are particularly surprising since this is a widely used model for investigating the mechanisms involved in ovulation in the adult. Our studies were carried out at a time corresponding to the morning of pro-oestrous. In the 4-day cycling adult rat, it is well-established (see Aiyer et al, 1974) that the response of the anterior pituitary to a single injection of GnRH increases by at least 10-fold from dioestrous through to the afternoon of pro-oestrous. This change in sensitivity is thought to be an important factor in controlling the magnitude of the pre-ovulatory LH surge. The subsensitive response of the PMSG-primed pituitary therefore appears somewhat paradoxical. Further, we have evidence (de Ziegler, Wilkinson, Cassard and Ruf, manuscript in preparation) that a similar situation exists in the intact animal; a single injection of GnRH given to "pro-oestrus" PMSG rats results in a severely blunted response of LH and FSH secretion.

It is of note that unlike the oestrogen primed animals (Caligaris et al, 1972) the two other methods of inducing an LH surge i.e. PMSG and brain stimulation, are characterised by high plasma levels of gonadotrophin in the 24 h immediately following treatment (Sashida and Johnson, 1976; YoungLai, Holmes and Ruf, 1976; Moll, Meijs-Roelofs, Kraemer and Dullaart, 1976). It is not known whether this fact in itself could be responsible for a diminished response to GnRH stimulation, possibly by a short-loop feedback of the gonadotrophins onto the pituitary. It is known, however, that one striking result of PMSG treatment is a precipitate fall, within 8 h (i.e. 40 hr before the LH peak), in the pituitary and hypothalamic concentration of cytoplasmic oestradiol receptors (Parker, Costoff, Muldoon and Mahesh, 1976). These levels remain low until after ovulation. During the normal oestrus cycle, a similar depletion of receptors occurs but with a shorter time period i.e. 20 h before the LH peak (Greeley, Muldoon and Mahesh, 1975). The drop in receptor population during the normal oestrus cycle coincides with increased pituitary response to GnRH stimulus. Thus, in the case of PMSG treatment, an extended reduction in oestradiol receptor levels appears able to reduce this responsiveness. It is possible that a similar situation occurs after brain stimulation. The results (Fig. 6) obtained from animals sacrificed 60 min. after the lesion suggest that this inhibition is at least partially present even at this early stage. It is also pertinent to note that this lack of response manifests itself only for FSH release, and may account in part for the shift in the plasma LH/FSH ratio previously described (Ruf et al, 1976). That a sham lesion can have a similar, though less pronounced acute effect, argues in favour of the idea that the tissue damage inflicted by the lesion/electrode releases sufficient GnRH to stimulate the anterior pituitary (Ruf et al, 1975).

In conclusion, the in vitro method we have described is a convenient and sensitive method for studying pituitary sensitivity to GnRH stimulation. It has allowed us to demonstrate that the pituitary of the immature female rat possesses control mechanisms for LH and FSH secretion similar to those observed in the adult and are present considerably before the onset of puberty.

REFERENCES

- Aiyer, M.S. , Fink, G. , and Greig, F. (1974). Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor during the oestrous cycle of the rat. *Journal of Endocrinology* 60, 47-64.
- Aiyer, M.S. , Sood, M.C. , and Brown-Grant, K. (1976). Pituitary response to exogenous luteinizing hormone releasing factor in steroid-treated gonadectomised rats. *Journal of Endocrinology* 69, 255-262.
- Apfelbaum, M. E. , and Taleisnik, S. (1976). Interaction between oestrogen and gonadotrophin-releasing hormone on the release and synthesis of luteinizing hormone and follicle-stimulating hormone from incubated pituitaries. *Journal of Endocrinology* 68, 127-136.
- Barracclough, C.A. , and Turgeon, J.L. (1975). Ontogeny of development of the hypothalamic regulation of gonadotrophin secretion : Effects of perinatal sex steroid exposure. In "The developmental biology of reproduction", pp. 255-273. Eds. C.L. Markett and J. Papaconstantinou. Academic Press, London.
- Ben-Jonathan, N. , Mical, R.S. , and Porter, J.C. (1974). Transport of LRF from CSF to hypophyseal portal and systemic blood and release of LH. *Endocrinology* 95, 18-25.
- Caligaris, L. , Astrada, J.J. , and Taleisnik, S. (1972). Influence of age on the release of luteinizing hormone induced by oestrogen and progesterone in immature rats. *Journal of Endocrinology* 55, 97-103.
- Cooper, K.L. , Fawcett, C.P. , and McCann, S.M. (1974). Inhibitory and facilitatory effects of estradiol-17 β on pituitary responsiveness to a luteinizing hormone-follicle stimulating hormone releasing factor (LH-RF/FSH-RF) preparation in the ovariectomized rat. *Proceedings of the Society for Experimental Biology and Medicine* 145, 1422-1426.
- de la Cruz, A. , Coy, D.H. , Vilchez-Martinez, J.A. , Arimura, A. , and Schally, A.V. (1976). Blockade of ovulation in rats by inhibitory analogs of LH-RH. *Science* 191, 195-197.
- Dowd, A.J. , Barofsky, A. -L. , Chaudhuri, N. , Lloyd, C.W. , and Weisz, J. (1975). Patterns of LH and FSH release from perfused rat pituitaries in response to infusions of hypothalamic extract. *Endocrinology* 96, 243-252.
- Drouin, J. , and Labrie, F. (1976). Selective effects of androgens on LH and FSH release in anterior pituitary cells in culture. *Endocrinology*, 98, 1528-1534.

- Drouin, J., Veilleux, R., Normand, M., and Labrie, F. (1975). Feedback effect of testosterone and estradiol on the LH responsiveness to LH-RH in anterior pituitary cells in culture. *Proceedings of the Canadian Federation of Biological Sciences* 18, 144, (Abstr. 574).
- Edwardson, J.A., and Gilbert, D. (1976). Application of an in vitro perfusion technique to studies of luteinizing hormone release by rat anterior hemipituitaries: Self-potential by luteinizing hormone releasing hormone. *Journal of Endocrinology* 68, 197-207.
- Ferland, L., Borgeat, P., Labrie, F., Bernard, J., de Lean, A., and Raynaud, J.-P. (1975). Changes of pituitary sensitivity to LH-RH during the rat estrous cycle. *Molecular and Cellular Endocrinology* 2, 107-115.
- Greeley, G.H., Muldoon, T.G., and Mahesh, V.B. (1975). Correlative aspects of LH-RH sensitivity and cytoplasmic estrogen receptor concentration in the anterior pituitary and hypothalamus of the cycling rat. *Biology of Reproduction* 13, 505-512.
- Hobson, W., and Hansel, W. (1974). Increased in vitro pituitary responsiveness to LH-RH after in vivo estrogen treatment. *Proceedings of the Society for Experimental Biology and Medicine* 146, 470-474.
- Kilpatrick, M.J., Collins, W.P., and Newton, J.R. (1976). Studies on the release of gonadotrophins during the superfusion of isolated rat pituitaries in a continuous flow system. *Journal of Reproduction and Fertility* 46, 25-30.
- Klein, D.C., and Weller, J. (1970). Input and output signals in a model neural system: the regulation of melatonin production in the pineal gland. *In Vitro* 6, 197-204.
- Legan, S.J., Coon, G.A., and Karsch, F.J. (1975). Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology* 96, 50-56.
- MacLusky, N., Chaptal, C., and McEwen, B. (1975). Postnatal ontogeny of cytoplasmic and nuclear estrogen-specific binding in the brain and pituitary of the rat. *Neuroscience Abstracts* 1, 439 (Abstr. 682).
- Martin, J.E., and Klein, D.C. (1976). Melatonin inhibition of the neonatal pituitary response to luteinizing-hormone-releasing factor. *Science* 191, 301-302.
- Moll, J., Meijs-Roelofs, H.M.A., Kramer, P., and Dullaart, J. (1976). Effects of electrical stimulation and of direct current and high frequency lesions of the hypothalamus on gonadotrophin release and puberty in female rats. *Annales de Biologie Animale, Biochimie, Biophysique* 16, 433-442.
- Osland, R.B., Gallo, R.V., and Williams, J.A. (1975). In vitro release of LH from anterior pituitary fragments superfused with constant or pulsatile amounts of LH-RH. *Endocrinology* 96, 1210-1216.
- Park, K.R., Saxena, B.B., and Gandy, H.M. (1976). Specific binding of LH-RH to the pituitary gland during the oestrous cycle in the rat. *Acta Endocrinologica* 82, 62-70.

- Parker, C.R., Costoff, A., Muldoon, T.G., and Mahesh, V.B. (1976). Actions of PMSG in the immature female rat : correlative changes in blood steroids, gonadotrophins and cytoplasmic estradiol receptors of the anterior pituitary and hypothalamus. *Endocrinology* 98, 129-138.
- Redding, T.W., and Schally, A.V. (1973). Distribution half-life and excretion of tritiated LH-RH in rats. *Life Sciences* 12, 23-
- Ruf, K.B., YoungLai, E.V., and Holmes, M.J. (1974). Induction of precocious sexual maturation in female rats by electrochemical stimulation of the brain. *Brain Research* 78, 437-446.
- Ruf, K.B., Wilkinson, M., and de Ziegler, D. (1975). Brain lesions and precocious puberty in rats. *Nature* 257, 404-405.
- Ruf, K.B., Kitchen, J.H., and Wilkinson, M. (1976). Synergistic effects of oestrogen and brain stimulation on precocious sexual maturation in the female rat. *Acta Endocrinologica* 82, 225-237.
- Sashida, T., and Johnson, D.C. (1976). Response of the immature rat ovary to gonadotrophins : acute changes in cyclic AMP, progesterone, testosterone, androstenedione and oestradiol after treatment with PMSG or FSH + LH. *Acta Endocrinologica* 82, 413-425.
- Schafer, S.J., and McShan, W.H. (1974). Gonadotrophic hormone release from fetal and adult rat pituitary glands after in vitro exposure to synthetic LH-FSH-RH. *Neuroendocrinology* 16, 332-341.
- Schally, A.V., Redding, T.W., and Arimura, A. (1973). Effect of sex steroids on pituitary responses to LH- and FSH-releasing hormone in vitro. *Endocrinology* 93, 893-902.
- Spona, J. (1974). LH-RH interaction with the pituitary plasma membrane is affected by sex steroids. *FEBS Letters* 39, 221-224.
- Spona, J. (1975a). LH-RH interactions with pituitary receptors : Properties and characterization. *Endocrinologica Experimentalis* 9, 127-134.
- Spona, J. (1975b). Sex steroids influence LH-RH receptor interaction. *Endocrinologica Experimentalis* 9, 167-176.
- Steinberger, A., and Chowdhury, M. (1974). Effect of testosterone and estradiol on the basal and LRF-stimulated secretion of gonadotrophins in pituitary cell culture. *Endocrine Research Communications* 1, 389-401.
- Tang, L.K.L., and Spies, H.G. (1975). Effects of gonadal steroids on the basal and LRF-induced gonadotrophin secretion by cultures of rat pituitary. *Endocrinology* 96, 349-356.
- Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R. (1972). Culture of enzymatically dispersed anterior pituitary cells : functional validation of a method. *Endocrinology* 91, 562-572.

- Vilchez-Martinez, Y.A., Arimura, A., Debeljuk, L., and Schally, A.V. (1974). Biphasic effect of estradiol benzoate on the pituitary responsiveness to LH-RH. *Endocrinology* 94, 1300-1303.
- Wilkinson, M., de Ziegler, D., and Ruf, K.B. (1976). Facilitatory effect of estradiol on luteinizing hormone secretion from immature female rat pituitaries in vitro. *Journal of Endocrinology* 70, 155-156.
- Wilson, C.A., Horth, C.E., Endersby, C.A., and McDonald, P.G. (1974). Changes in plasma levels of oestradiol, progesterone and luteinizing hormone in immature rats treated with pregnant mare serum gonadotrophin. *Journal of Endocrinology* 60, 293-304.
- YoungLai, E.V., Holmes, M.J., and Ruf, K.B. (1976). Changes in concentration of LH, FSH, and estrogen in the immature female rat during precocious sexual maturation induced by electrochemical stimulation of the brain. *Hormone Research*. In press.
- Zeballos, G., and McCann, S.M. (1975). Alterations during the estrous cycle in the responsiveness of the pituitary to subcutaneous administration of synthetic LH-RH. *Endocrinology* 96, 1377-1385.
- de Ziegler, D., Wilkinson, M., and Ruf, K.B. (1976). Precocious puberty in female rats : on the mode of action of hypothalamic lesions. *Annales de Biologie animale, Biochimie, Biophysique* 16, 443-451.

Table 1

Treatment	Time of incubation with GnRH	LH secretion ng/ml/60 min		FSH secretion ng/ml/60 min	
		1st stimulation	2nd stimulation	1st stimulation	2nd stimulation
OB	15 min	N.S. 9296 $\pm$ 679 (12)	N.S. 10100 $\pm$ 583 (6)	3444 $\pm$ 270 ⁰ (10)	N.S. 3830 $\pm$ 307 (6)
	60 min	8844 $\pm$ 800 (10)	10200 $\pm$ 1100 (5)	2548 $\pm$ 193 (12)	3403 $\pm$ 373 (5)
	15 min	*** 2658 $\pm$ 325 (12)	*** 3428 $\pm$ 427 (6)	** 2896 $\pm$ 192 (12)	*** 1455 $\pm$ 86 (6)
Oil	60 min	5285 $\pm$ 630 (10)	7930 $\pm$ 986 (5)	2096 $\pm$ (10)	2811 $\pm$ 322 (4)

Comparison of two consecutive 15 min pulses of GnRH with two 60 min stimulations with GnRH.
Hemipituitaries from 25 day old rats treated overnight with OB or oil.

() indicates number of cultures ; statistics refer to differences between 15 min and 60 min incubations within each group ($\pm$ S. E. M.)

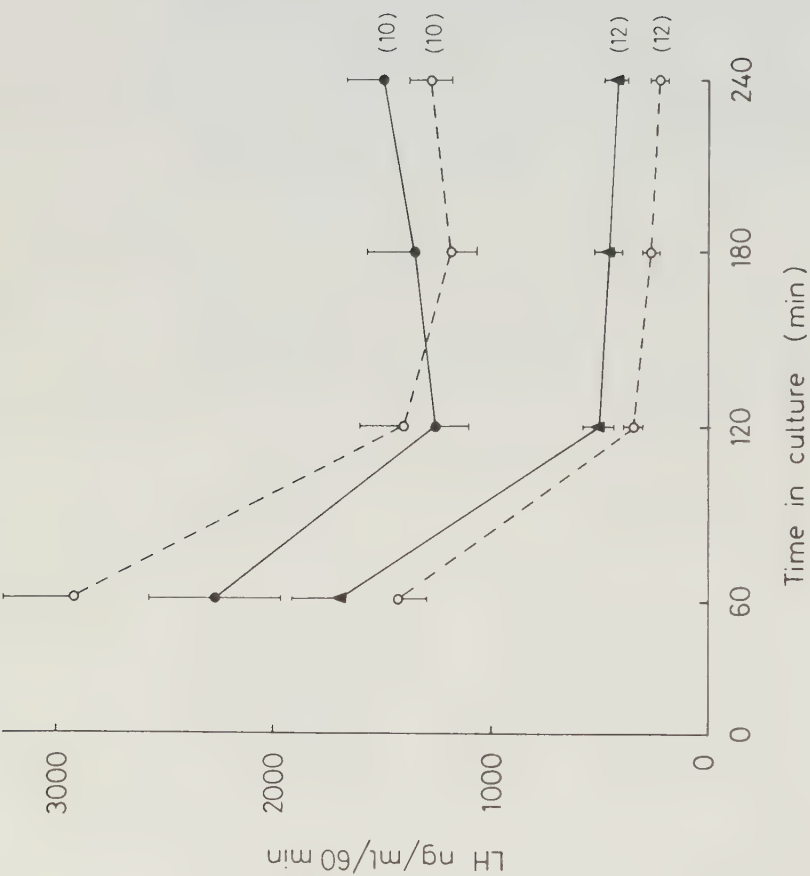


Fig. 1 Basal (i.e. unstimulated) secretion of LH from hemipituitaries removed from OB treated (1 injection; ●—●) and untreated (○—○) animals aged 25 days (upper two lines) and 12 days (▲ and ○). The values of LH (ng/ml; $\pm$ S.E.M.) represent total LH secreted into medium during each of four consecutive 60 min incubations. The figures refer to the number of cultures.

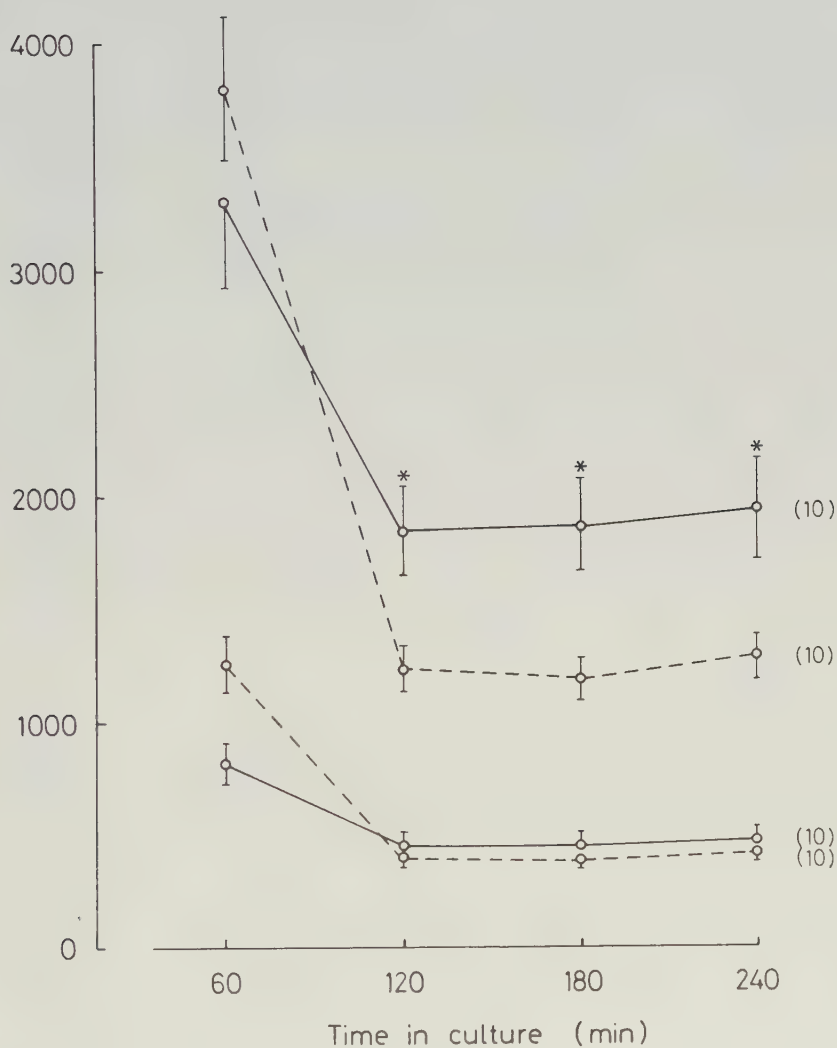


Fig. 2 Basal secretion of LH from hemipituitaries removed from OB treated (2 injections ; ●—●) and untreated (o---o) animals aged 26 days. The upper two lines are expressed as ng/ml/60 min of LH ; the lower two are converted to ng/mg tissue/60 min. Each point (12 cultures) represents the quantity of LH released in each of four consecutive 60 min incubations.

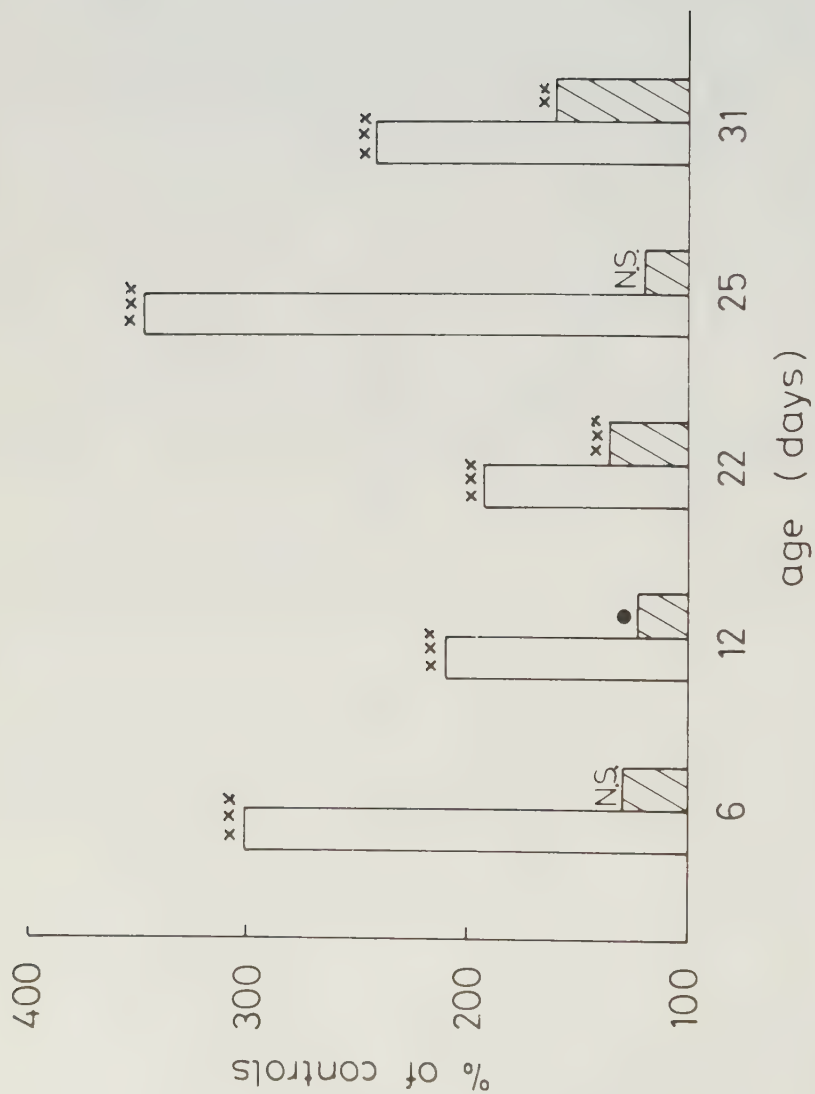


Fig. 3 Increases in LH and FSH release after a single injection of OB, as percentages of controls, following a 15 min pulse of GnRH. Control values (i. e. stimulation of control hemipituitaries) given in ng/ml/60 min ($\pm$ S. E. M.).

□, LH ▨, FSH.

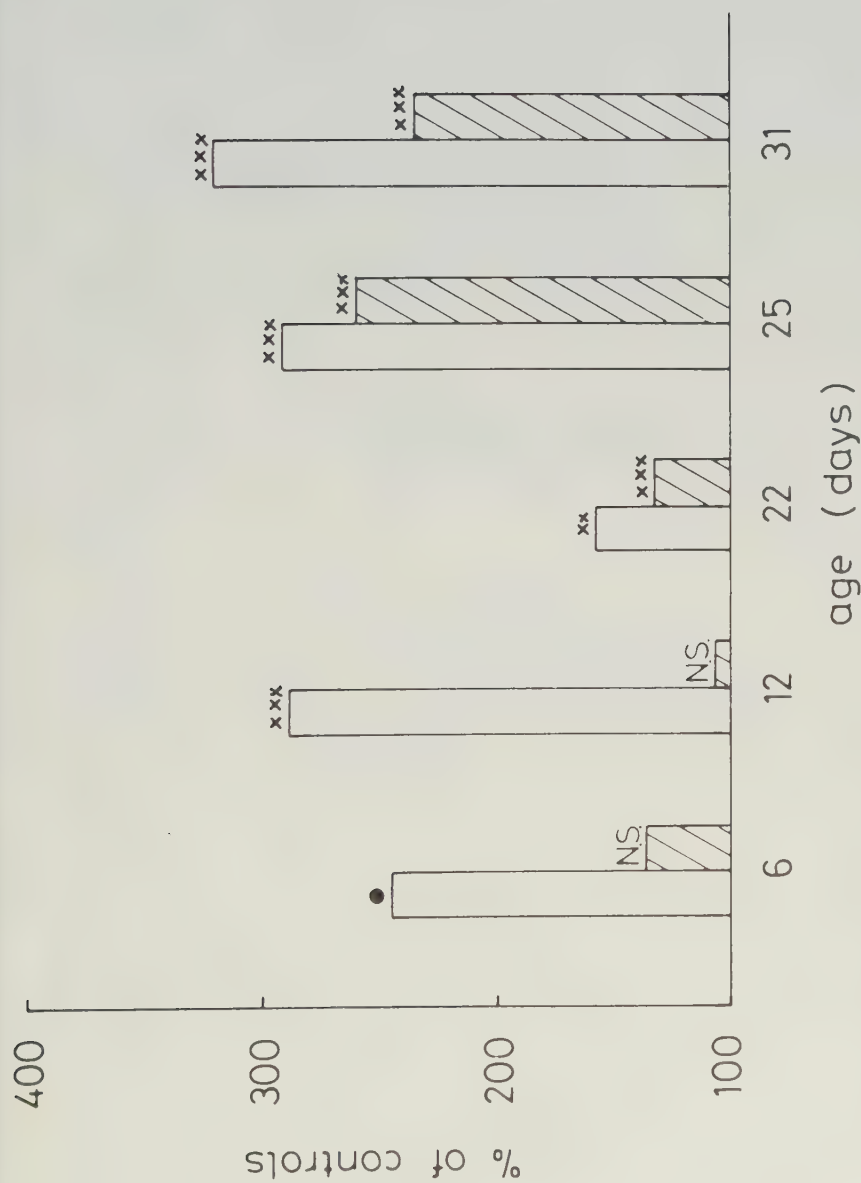


Fig. 4 As for Fig. 3, increases in LH and FSH release, as percentages of controls, after a second pulse of GnRH.

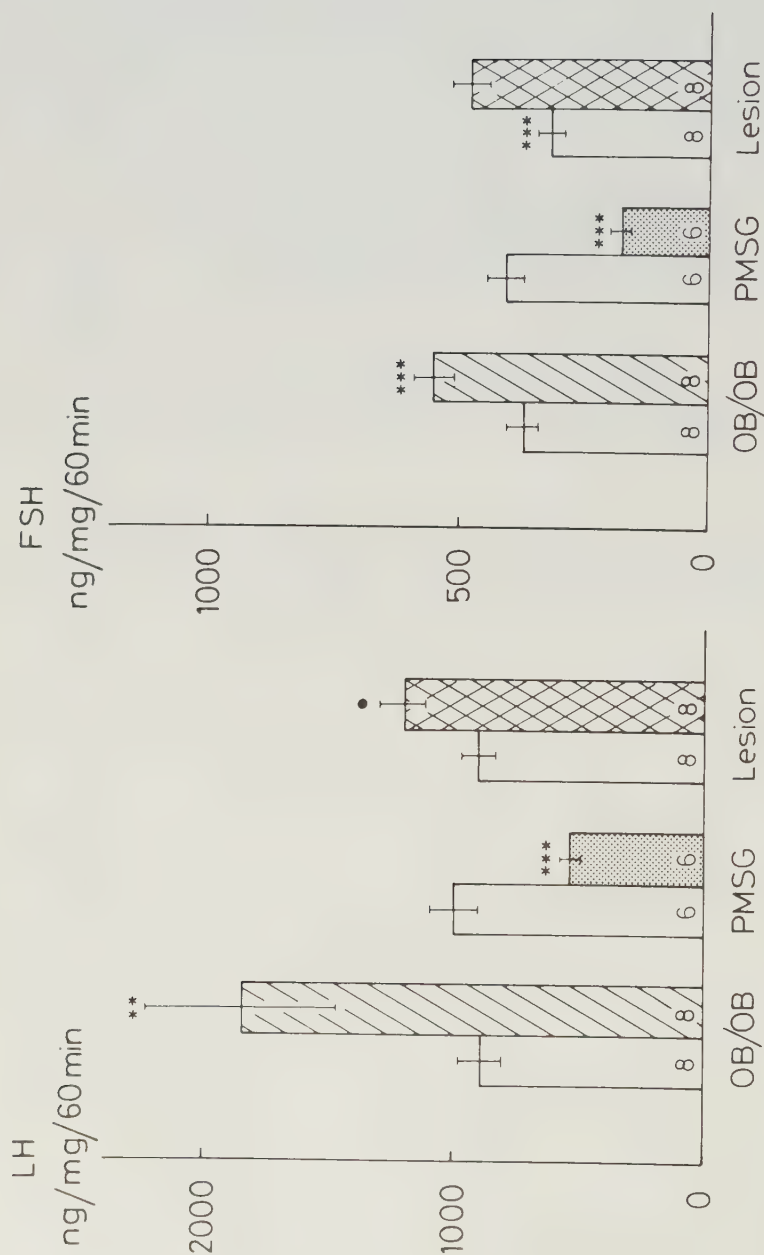


Fig. 5 Release of LH and FSH (ng/mg tissue/60 min, $\pm$ S.E.M.) after a single 15 min pulse of GnRH; comparison of three pretreatments, OB/OB (two injections of OB; days 23 and 25); injection of PMSG (10 i.u.; day 24); brain lesion (day 23). Stimulations carried out on day 26. □, controls; ▨, OB/OB; ▨, PMSG; ▨, brain lesion. Number indicates numbers of cultures.

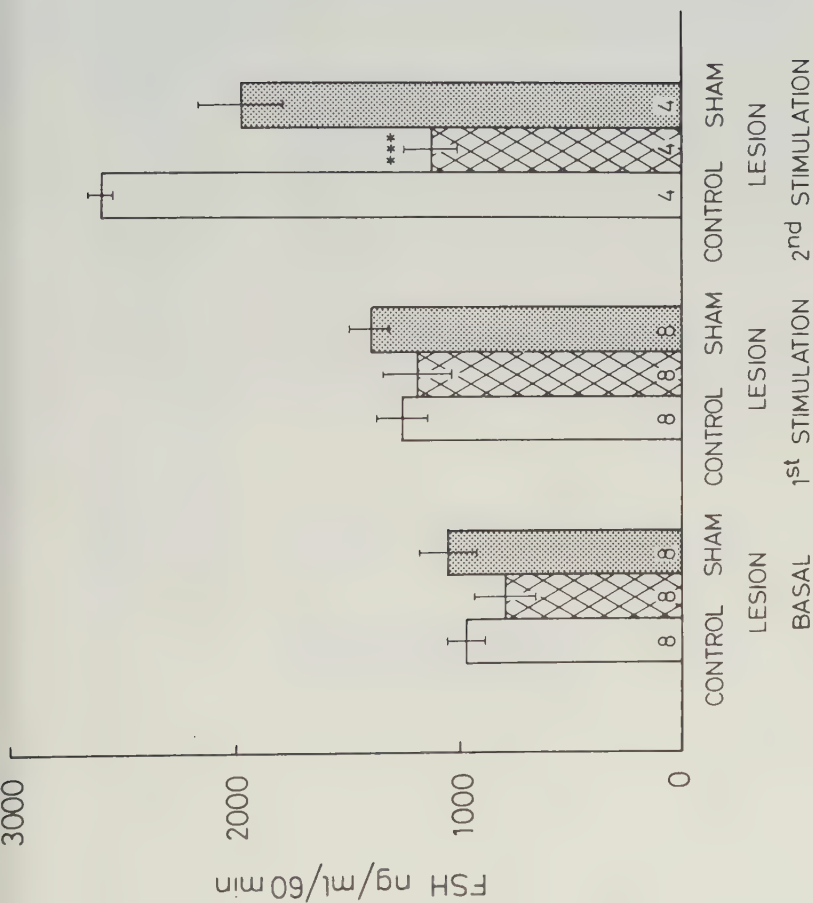


Fig. 6 Acute effects of brain lesion on basal release of FSH (ng/ml/60 min + S. E. M.) and release after two consecutive (60 min) pulses, of GnRH. $\square$ controls; $\otimes$ lesioned; ▨ sham lesioned. Animals aged 23 days.

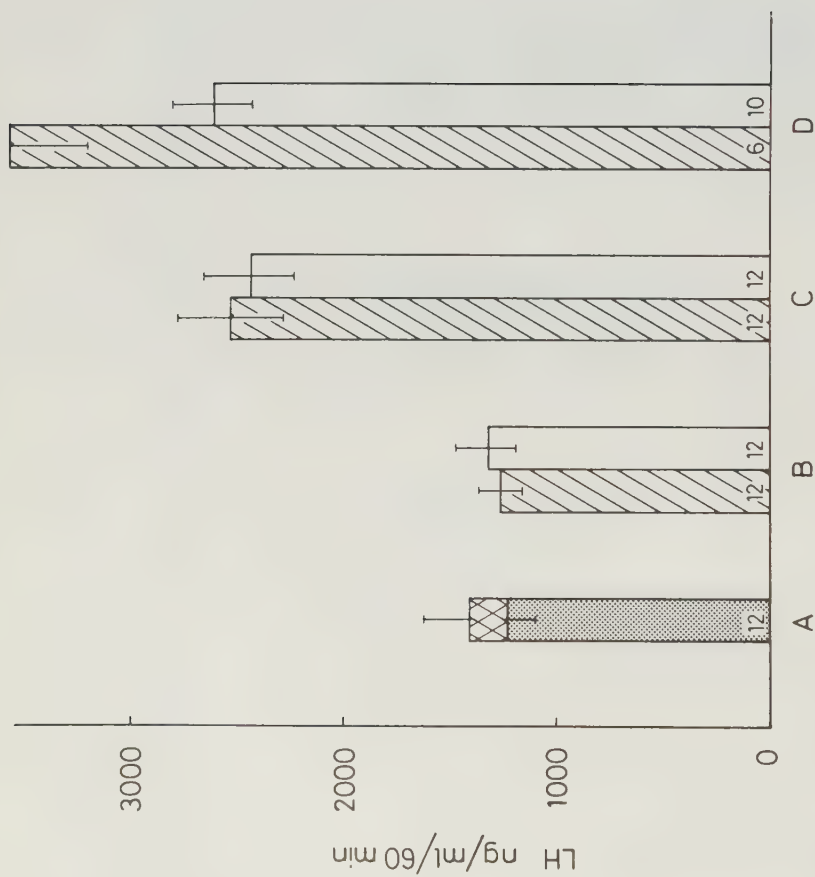


Fig. 7 Effect of overnight (16 h) incubation of hemipituitaries (from 24 day old rats) with OB (10^{-9} mol/l). A and B compare basal secretion of LH (ng/ml/60 min; $\pm$ S.E.M.) obtained after priming with OB in vivo (A) with that in vitro (B). C and D show LH secretion after two consecutive pulses of GnRH. $\boxtimes$, basal release after 16 h priming in vivo and 2 h in vitro; $\boxdot$, controls; $\boxminus$, 16 h priming in vitro; $\square$, in vitro controls.

CHAPTER III

ANTERIOR PITUITARY SENSITIVITY IN IMMATURE FEMALE
RATS STIMULATED IN VIVO WITH GONADOTROPHIN RELEASING
HORMONE : EFFECT OF PRIMING WITH OESTROGEN, PMSG OR
BRAIN LESIONS

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I. SUMMARY

An investigation of pituitary sensitivity (PS) to stimulation with GnRH was carried out in 26 day-old immature female rats.

We have compared PS (in terms of plasma LH and FSH increments) to one or two injections of the polypeptide in animals which had received one of the following priming treatments : oestradiol benzoate (OB) (10 μ g) administered either as a single injection on day 23 (OB/-), or on day 25 (-/OB), or on both days (OB/OB) ; pregnant mare serum gonadotrophin (PMSG) (10 i.u.) on day 24 ; an electrochemical brain lesion (✂) placed in the mediobasal hypothalamus on day 23 ; control animals (C) received either vehicle alone or a sham lesion. OB pretreatment enhanced PS, assessed at 1000 h on day 26, after one or two injections of GnRH (100 ng/100 g body weight ; s.c.) similarly in the three groups (OB/-, -/OB, OB/OB) in terms of LH ($p < 0.01$). The FSH response was also consistently increased after OB treatment, but these increments failed to reach statistical significance.

In contrast, 48 h after the injection of PMSG, i.e. in a "pro-oestrous-like" condition, PS was sharply decreased for both LH and FSH ($p = 0.001$). In lesioned animals, PS to one injection of GnRH was unchanged. A second GnRH injection applied after a 60 min interval induced a slightly larger LH response in controls. In contrast, the ratio of the second response to the first is increased in PMSG animals, despite the state of overall decrease in PS, being 4.5:1 in PMSG, versus 1.4:1 in C.

In a second set of experiments, we investigated the variation of PS in conjunction with an experimentally induced gonadotrophin surge. In animals of the OB/- group additionally treated with progesterone (1 mg) at 1200 h on day 26, PS was increased at both 1400 and 1700 h as compared with PS in the OB/- group at 1000 h. PMSG-treated animals maintained their state of decreased responsiveness at 1400, but exhibited increased PS at the time of the gonadotrophin surge (1700 h).

These results show that OB increases PS to GnRH in 26 day-old female rats and that the induction of a gonadotrophin surge further increases this sensitivity. In contrast, PMSG-treated rats display a state of decreased responsiveness 48 h and 52 h, but not 55 h after the injection. PS observed on the second day after PMSG treatment thus clearly differs from that observed during pro-oestrus in the adult cycling female rat.

II. INTRODUCTION

In the female rat the sensitivity of the anterior pituitary gland to gonadotrophin releasing hormone (GnRH) varies through the reproductive cycle, displaying a sharp increase at the time of the preovulatory gonadotrophin surge (Cooper, Fawcett and McCann, 1974 ; Aiyer, Fink and Greig, 1974 a ; Ferland, Borgeat, Labrie, Bernard, de Lean and Raymond, 1975 ; Zeballos and McCann, 1975). The pro-oestrous increase in responsiveness of the gland is a two-step process (Aiyer, Chiappa and Fink, 1974b). The first phase takes place in the morning and early afternoon of pro-oestrus. It is believed to be dependent on the high plasma oestrogen levels normally found at this time (Aiyer and Fink, 1974). Later in the afternoon of pro-oestrus, when GnRH levels in pituitary stalk blood are elevated (Fink, 1976), pituitary sensitivity to exogenous GnRH stimulation is further increased. This second phase is probably the result of a priming effect of the GnRH, so that further stimulation results in a greater response (Aiyer et al, 1974b). In vivo treatment of adult met-oestrous or ovariectomized female rats with exogenous oestradiol benzoate (OB) increases the sensitivity of the pituitary to stimulation with GnRH both in vivo (Cooper et al, 1974 ; Vilchez-Martinez, Arimura, Debeljuk and Schally, 1974 ; Aiyer and Fink, 1974 ; Aiyer, Sood and Brown-Grant, 1976) and in vitro (Hobson and Hansel, 1974 ; Apfelbaum and Taleisnik, 1976). This enhancement only becomes apparent 6 hours after the steroid injection (see for example Wilchez-Martinez et al, 1974) ; it is preceded by an inhibitory phase during which pituitary responsiveness is decreased compared to untreated controls. It appears, from recent work, that oestrogen added to pituitary tissue cultures (Labrie, 1976) increases the stimulatory effect of GnRH at the pituitary itself. In an earlier in vitro study we investigated the ontogeny of the oestrogen effect on pituitary responsiveness to GnRH and reported that OB pretreatment increased the sensitivity of the gland to GnRH stimulation as early as day 5 of life (Wilkinson, de Ziegler and Ruf, 1976). In the present work we have extended these studies to the intact immature female rat and have investigated the effect of various types of oestrogen priming on the response of the pituitary to injected GnRH. In addition, we have studied pituitary sensitivity at different phase of an induced gonadotrophin surge. Priming with exogenous oestrogen was achieved with one or two injections of OB. In other experimental groups endogenous oestrogen levels were increased through stimulation of the reproductive system e.g. by treatment with pregnant mare serum gonadotrophin (PMSG) (Wilson, Horth, Endersby and McDonald, 1974) or placement of a brain lesion in the basal hypothalamus (Ruf, YoungLai and Holmes, 1974 ; de Ziegler, Wilkinson and Ruf, 1976).

III. MATERIALS AND METHODS

Female rats (Sprague-Dawley derived SIV 50) of certified age were obtained on day 21 and caged in groups of 8 under conditions of controlled illumination (lights on from 0700-1900 h) and temperature ($22 \pm 1^\circ \text{C}$). On day 26 the responsiveness of the anterior pituitary to stimulation with GnRH (Beckman ; 100 ng/100 g body weight ; 0.2 ml, 0.9 % saline ; s.c.) was tested in variously primed animals (see below). The response was evaluated in terms of plasma LH and FSH at 30 and 90 min after the injection. In some experiments, two injections of GnRH were used, separated by 60 min, and the rats sacrificed 30 min after the second. Blood was collected by decapitation and plasma stored at -30°C for gonadotrophin assay. LH and FSH were determined by double antibody radioimmunoassay using kits supplied by NIH. Results are expressed in terms of LH and FSH NIAMDD-RP-1 standards. Statistical analysis was by unpaired Student's t-test. *** $p < 0.001$; ** $p < 0.005$; * $p < 0.01$; • $p < 0.025$.

Preparation of animals

Rats were primed in various ways and were all used on day 26 of life. The oestrogenic effect of the different priming methods was assessed by increases in uterine weight.

a) Single injection of OB (-/OB). This group received a single injection (10 μg ; 0.1 ml of peanut oil ; s.c.) at noon on day 25 of life.

b) Two injections of OB (OB/OB). This group received two injections of OB (10 μg , 0.1 ml of oil ; s.c.) at noon on days 23 and 25 of life, according to the model described by Caligaris, Astrada and Taleisnik (1972).

c) Injection of OB followed by progesterone (OB/P). In a modification of (b) above, the second OB injection was omitted and replaced by one of progesterone (1 mg in oil, 0.1 ml, s.c.) on day 26 (noon) ; (Caligaris et al, 1972). Experiments performed in the morning of day 26, i.e. before the administration of progesterone, are referred to as OB/-

d) Priming with PMSG. Animals received an injection of PMSG (Gestyl^(R) Organon ; 10 i.u., 0.2 ml saline ; s.c.) at 1000 h on day 24 of life. Day 26 was then technically pro-oestrus.

e) Electrochemical lesion of the hypothalamus ($\frac{1}{2}$). Performed on animals 23 days of age, a direct anodal current (0.5 mA ; 15 sec) was passed through a stainless steel electrode whose uninsulated tip was stereotaxically placed near the origin of the pituitary stalk (Ruf et al, 1974).

f) Control group (C). These received injections of vehicle only, or a sham lesion.

A longitudinal study of pituitary sensitivity after brain lesions was carried out by injecting GnRH intravenously (100 ng ; 0.1 ml, saline) at 1000 h on each day following the lesion. Under ether anaesthesia, 0.8 ml of blood was collected before injecting GnRH. A second blood sample was obtained 10 min later via aortic puncture.

IV. RESULTS

Effects of the different priming treatments on uterine weight

All treatments used are known to elevate plasma oestrogen levels. We have taken uterine weights to be a good index of the oestrogenic effect. The results appear in Table 1. Day 26 of life is approximately 9 days before the normal onset of puberty in our colony. Brain lesions performed on day 23 (4) result in a uterine enlargement on day 26 comparable to that observed on the same day after 10 µg of OB have been injected on day 25. Steroid treatments (OB/-, OB/OB and OB/P) as well as PMSG, result in larger uterine responses with signs of fluid retention.

Effects of a single or repeated GnRH injection in 26-day-old immature female rats

Plasma LH and FSH were determined before, and 30 or 90 min after one or two injections of GnRH (100 ng/100 g b.w. ; s.c.). The injections were carried out between 1000 and 1100 h in 26 day-old rats. The results are illustrated in Fig. 1a and 1b : in unprimed animals (C) a single injection of GnRH resulted in a significant increase above basal plasma values of both LH and FSH (respectively $p < 0.001$, and $p < 0.01$). This result is comparable to that obtained with adult rats after a subcutaneous injection of GnRH. Subcutaneous injections mimic intravenous infusions of the decapeptide (Zeballos and McCann, 1974) and induce a rise in both gonadotrophins. A second GnRH injection given 60 min after the first resulted in a moderate, but significantly larger ($p < 0.025$) elevation in plasma LH. In terms of FSH, the response is also slightly higher than that observed after the first GnRH injection, though is not statistically different. This observation, made in unprimed female rats, may be compared to non-pro-oestrous adult females where self-priming of the anterior pituitary gland by GnRH to further stimulation is minimal and thus quite different to the condition encountered on pro-oestrus (Aiyer et al, 1974b). Both basal LH and FSH were uniformly low on the morning of day 26 in control and in experimental animals except in the case of the PMSG-treated group. In this particular case, basal plasma LH was significantly higher ($p < 0.025$) than controls where as FSH was significantly lower ($p < 0.005$). Pretreatment with OB (10 µg in oil ; s.c.) at noon the previous day (-/OB) resulted in a significant ($p < 0.01$) increase in LH release after GnRH stimulation, when compared with controls. This observation is in accord with the results of our experiments carried out in vitro (Wilkinson et al, 1976) as well as with data concerning either intact (Vilchez-Martinez et al, 1974) or ovariectomized (Aiyer, Sood and Brown-Grant, 1976) adult female rats. The FSH response after one OB injection is also increased, but not significantly so. After the second injection of GnRH, both gonadotrophins are elevated over control values.

The steroid treatment OB/OB was reported to trigger an LH surge on the afternoon of day 26 via a positive feedback effect (Caligiariş et al, 1972). In this model the sensitivity of the gland to one or two GnRH stimulations given in the morning of day 26 is not further increased when compared to the effect of a single OB injection (-/OB) despite the increased exposure to high plasma oestrogen levels resulting in higher uterine weights (Table 1).

In lesioned animals displaying signs of endogenous oestrogen production (i.e. increased uterine weight) the pituitary responsiveness to one GnRH injection was surprisingly unchanged when compared with that of the control group. The FSH increment was reduced but not significantly. After the second GnRH injection the increments are slightly higher than those found in controls. This unexpected result led us to investigate the responsiveness of the gland to GnRH in a longitudinal study. The brain lesion was performed on day 23 of life; the rise in plasma LH was evaluated 10 min after an intravenous injection of 100 ng of GnRH, given every morning starting on day 24. The results, compared with unlesioned animals of the same age, are shown in Table 2. Plasma LH increments observed in lesioned animals are smaller than in controls from day 24 to day 26, though the difference is not statistically significant. The injection of 10 i.u. of PMSG on day 24 induces within 72 h, i.e. on day 27, premature first ovulation in more than 90 % of the rats. We have tested pituitaries sensitivity on the morning of day 26, i.e. the preovulatory day corresponding to pro-oestrus (Costoff, Eldrige and Mahesh, 1974) and observed that the response to GnRH stimulation is strikingly depressed both for LH and FSH release. The increment in plasma LH after one injection of GnRH is minimal, being only 12 % ($p < 0.001$) of that observed in untreated controls and almost no plasma FSH increment, above an already depressed basal value, is observed 30 and 90 min after one injection. This result is in sharp contrast with that obtained in adult cycling rats on the day of pro-oestrus, when an increase in responsiveness is obtained. The increment in plasma LH induced in PMSG animals by a second GnRH injection is less pronounced than that observed in controls whilst an increase in FSH is completely absent. For LH, despite the decrease in overall responsiveness, the ratio of the second increment to the first is greater (4.5:1) in the PMSG rats than in controls (1.4:1).

Anterior pituitary sensitivity in relation to LH pre-injection values.

In the adult female rat a second phase in the increase in pituitary responsiveness to GnRH stimulation occurs during the afternoon of pro-oestrus. We have thus investigated pituitary sensitivity at different times of the day and in conditions where an LH surge has been induced. These results are compared with the GnRH effect observed in animals in which a surge of gonadotrophins is not induced i.e. in untreated rats, or in those receiving only one injection of OB. In a first series of experiments the increment in plasma LH after one and two injections of GnRH was determined at different times of the day, i.e. 1000, 1400 and 1700 h in animals which had received two OB injections (OB/OB). Comparisons were made with data obtained under the same conditions from animals injected with either vehicle only (Controls) or with a single dose of OB (-/OB) on day 25, which does not induce any LH peak in animals of this age (Caligiariş

et al, 1972). Increments in plasma LH are illustrated in Fig. 2 and plasma values listed in Table 3. In control animals the increment in plasma LH observed 30 min after a single GnRH injection was identical at all times tested ; sensitivity to the second injection however, appeared to increase during the afternoon. Pretreatment with a single OB injection on day 25 increases the responsiveness ($p < 0.01$) of the gland but this is not modified when investigated at different times through day 26. In the OB/OB group, the magnitude of the response to one and two GnRH injections was not significantly further increased over the -/OB group (Fig. 2). However, in our hands this steroid treatment did not lead to an LH surge (Table 3). Thus, no conclusion can be drawn as to the nature of the observed increase in sensitivity.

Alternative models were then used to induce a peak of LH in these 26 day-old female rats. Progesterone (1 mg at noon on day 26) was used in the OB/P group to induce an LH surge in OB-primed animals (10 μ g ; day 23) Caligaris et al, 1972). In addition, the PMSG model was used again to investigate the pituitary sensitivity at 1400 and 1700 h on the day preceding ovulation (pro-oestrus). Basal plasma LH and the elevation observed 30 min after GnRH injection are illustrated in Fig. 3. In OB/P rats, pituitary responsiveness was found to be increased at 1400 h, i.e. two hours after the progesterone injection, as compared with the sensitivity of OB/-primed animals (10 μ g OB on day 23) assessed at 1000 h (Fig. 3 ; $p < 0.02$). In OB/P rats, this increase in responsiveness occurs before basal LH values begin to rise. At 1700 h, when a massive LH surge takes place, pituitary sensitivity to GnRH stimulation is maximal but increments are not statistically larger when compared with the conditions observed at 1400 h. In PMSG-treated rats, as already stated, responsiveness of the gland tested in the morning of day 26 is small and significantly ($p < 0.001$) less than that of untreated controls of the same age. No modification is observed at 1400 h despite a slight rise in basal values. At 1700 h, basal plasma LH reached high levels comparable to the preovulatory surge encountered in adults and in contrast to the observation made in the morning, pituitary responsiveness to GnRH stimulation was greatly increased and represented 225 % of control values.

V. DISCUSSION

In previous *in vitro* studies (Wilkinson et al, 1976) we have followed the ontogeny of the sensitizing effect of oestrogen pretreatment on pituitary responsiveness to GnRH stimulation. An increase in responsiveness was observed as early as day 5 of life. In the present experiments we have extended this *in vitro* work to include an investigation of the effects of oestrogen pretreatment on pituitary sensitivity to subcutaneous injections of GnRH in the immature female rat. We have used the 26 day-old animal because this prepubertal age offers the advantages of comparing the effects on pituitary sensitivity of a single injection of oestrogen, which does not provoke a rise in gonadotrophins, with those resulting from manipulations inducing a gonadotrophin surge. This has been achieved by sequential steroid treatment with OB/OB, or OB/P, or by inducing the endogenous production of oestrogen by PMSG, or by brain lesion.

Pituitary sensitivity to injected GnRH is increased in the -/OB primed animals (i.e., OB, day 25) when compared with untreated controls (Fig. 1a and 1b), thus confirming our *in vitro* observations. The sequence of two OB injections (OB/OB) on days 23 and 25 accompanied by increased stimulation of uterine weight with massive fluid retention does not, however, further increase pituitary sensitivity on the morning of day 26 above that observed in the -/OB group (cf Fig. 1a and 1b). When the second injection is omitted (i.e. OB/-) the increase in pituitary responsiveness is comparable (cf Fig. 3). The oestrogenic increase in pituitary responsiveness tested on day 26 appears to be already maximal with a single injection of OB, given either on day 25 (-/OB) (Fig. 1a) or on day 23 (OB/-) (Fig. 3).

The experimental group tested with PMSG on day 24 is in a pseudo-pro-oestrous state by day 26, i.e. the plasma values of sex steroids and gonadotrophins resemble those seen on pro-oestrus in adult cycling rats (Costoff et al, 1974; Wilson et al, 1974; Parker, Costoff, Muldoon and Mahesh, 1976). In particular, the occurrence of an LH surge in the late afternoon is dependent, in both cases, on high plasma oestrogen (adult: Kalra, 1975; PMSG: Ferin, Zimmering and Vande Wiele, 1969). In view of this analogy, the results (Fig. 1a and 1b) observed in PMSG-treated animals were unexpected; pituitary responsiveness to one injection of GnRH is sharply decreased when compared to untreated controls both in terms of LH (22 % of controls; $p < 0.001$) and of FSH, where almost no elevation is seen. This observation made at 1000 h in the morning of day 26 can be repeated at 1400 h of the same day (Fig. 3). However, by 1700 h responsiveness to GnRH injection is recovered. At this time high basal LH values indicate the occurrence of the preovulatory LH surge (see Fig. 3) and the increment in plasma LH observed 30 min after GnRH injection greatly exceeds control values. This progressive change in pituitary sensitivity to GnRH stimu-

lation during the day preceding ovulation induced by PMSG, is markedly different from that which occurs in adult cycling females on the day of pro-oestrus. In the latter case pituitary sensitivity increases from the morning of pro-oestrus and extends to the time of the LH surge (Aiyer et al, 1974b). It is of note therefore that 48 h after PMSG injection, technically pro-oestrus, when oestrogen levels are high, an increase in pituitary sensitivity to GnRH stimulation is completely lacking until the LH surge occurs. Rather, a pronounced decrease in responsiveness of the gland is observed. The early oestrogen mediated increase in pituitary sensitivity is therefore not required for the LH surge to occur in this model. The origin of the decrease in pituitary sensitivity after PMSG injection is not clear. We have noted a similar lack of response of pituitaries from PMSG animals when tested *in vitro* (see chap. II) and suggested that the prolonged suppression of pituitary cytosol oestrogen receptors, reported by Parker et al (1976) could be partly responsible. These same authors (Parker et al, 1976) also show that PMSG treatment is quickly followed by a rise in serum testosterone which remains high until ovulation. Testosterone is known to inhibit the response of pituitary cells to stimulation with GnRH *in vitro* (Drouin and Labrie, 1976). Thus, the rise in levels and the reduction in oestrogen receptors could account for the drop in pituitary sensitivity after PMSG treatment, though the recovery of sensitivity (1700 h, pro-oestrus) takes place despite these two situations being unchanged.

A surge of LH was induced on the afternoon of day 26 by injecting OB-primed animals with progesterone, at noon the same day (OB/P group). It is interesting to compare these data with results observed in the PMSG animals. As already discussed, one injection of OB performed on day 23 increases pituitary sensitivity on the morning of day 26 to the same degree as in the -/OB and OB/OB groups. However, when GnRH is injected 2 h after the progesterone, i.e. at 1400 h, the increase in plasma LH is larger than that observed in the morning ($p < 0.02$), though basal LH values remain low. At 1700 h a massive LH surge is observed. Basal LH values approach 2000 ng/ml i.e. higher levels than those found during the PMSG-induced or normal preovulatory LH surges. The LH response of the pituitary gland to GnRH stimulation applied at this time is maximal (Fig. 3). Fink (1976) has recently reported that progesterone given at midday to oestrogen primed ovariectomized adults provokes, as long suspected, a rise in radioimmunoassayable GnRH in hypophyseal portal blood. It is thus probable that the endogenous release of GnRH artificially induced in these immature female rats is responsible, through a priming effect on the pituitary, for the increase in responsiveness observed at 1700 h. In this OB/P model (Caligaris et al, 1972) the pituitary sensitivity to GnRH stimulation follows the same sequence of events as described in the adult pro-oestrous state (Aiyer et al, 1974a) but in this experimental design, which uses non-physiological amounts of steroids, the changes are probably exaggerated.

Pituitary responsiveness of lesioned animals to one GnRH injection is either unchanged (Fig. 1a and 1b) or decreased (Table 2) when compared with that of controls. Ovarian content of testosterone has been found to be increased in lesioned animals (Ruf, unpublished data) together with the increase in plasma and ovarian oestrogen (YoungLai, Holmes and Ruf, 1976). It is thus possible

that testosterone antagonises the effect of oestrogen at the pituitary level as earlier discussed in the case of PMSG.

In conclusion, these results confirm and extend our *in vitro* studies. The observations made in the PMSG model were unexpected, and suggest that an increase in sensitivity of the pituitary to GnRH, prior to the LH peak, may not be necessary for a full preovulatory LH surge, and ovulation to take place. This is not to say that the pituitary is not affected by oestrogen in the PMSG model. On the morning of day 26 the ratio of the LH response after a second injection of GnRH to that induced by the first is larger in PMSG animals than in untreated controls (4.5:1 versus 1.4:1) (Fig. 1a). The ability of GnRH to prime the pituitary to further stimulation as observed in PMSG animals may be the result of an effect on the gonadotrophs mediated, directly or indirectly, by oestrogen which is probably important for the LH surge to take place. Results obtained in the PMSG model show that this may happen without an overall increase in the gland responsiveness to GnRH. It is also interesting to note that an LH surge can be induced in oestrogen-primed ovariectomised rats without any concomitant change (positive or negative) in pituitary sensitivity (Legan and Karsch, 1975). Nevertheless, the differences observed between preovulatory pituitary sensitivity in the PMSG-treated animal and that in the adult call for caution in the use of PMSG as a model of the oestrous cycle despite its obvious advantages.

VI. REFERENCES

- Aiyer, M.S. and Fink, G. (1974). The role of sex steroid hormones in modulating the responsiveness of the anterior pituitary gland to luteinizing hormone releasing factor in the female rat. *Journal of Endocrinology* 62, 553-572.
- Aiyer, M.S., Fink, G. and Greig, F. (1974a). Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor during the oestrous cycle of the rat. *Journal of Endocrinology* 60, 47-64.
- Aiyer, M.S., Chiappa, S.A. and Fink, G. (1974b). A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat. *Journal of Endocrinology* 62, 573-588.
- Aiyer, M.S., Sood, M.C. and Brown-Grant, K. (1976). The pituitary response to exogenous luteinizing hormone releasing factor in steroid treated gonadectomized rats. *Journal of Endocrinology* 69, 255-262.
- Apfelbaum, M.E. and Taleisnik, S. (1976). Interaction between oestrogen and gonadotrophin-releasing hormone on the release and synthesis of luteinizing hormone and follicle-stimulating hormone from incubated pituitaries. *Journal of Endocrinology* 68, 127-136.
- Caligaris, L., Astrada, J.J. and Taleisnik, S. (1972). Influence of age on the release of luteinizing hormone induced by oestrogen and progesterone in immature rats. *Journal of Endocrinology* 55, 97-103.
- Cooper, K.J., Fawcett, C.P. and McCann, S.M. (1974). Variation in pituitary responsiveness to a luteinizing hormone/follicle-stimulating hormone-releasing factor (LH-RF/FSH-RF) preparation during the rat estrous cycle. *Endocrinology* 95, 1293-1299.
- Costoff, A., Eldridge, J.C. and Mahesh, V.B. (1974). Pituitary ultra-structure and serum gonadotrophin levels in the PMS-primed immature rat. *Cell and Tissue Research* 151, 79-92.
- Drouin, J. and Labrie, F. (1976). Selective effect of androgens on LH and FSH release in anterior pituitary cells in culture. *Endocrinology* 98, 1528-1534.
- Ferin, M., Tempone, A., Zimmering, P.E. and Vande Wiele, R.L. (1969). Effect of antibodies to 17β -estradiol and progesterone on the estrous cycle of the rat. *Endocrinology* 85, 1070-1077.
- Ferland, L., Borgeat, P., Labrie, F., Bernard, J., de Lean, A. and Raynaud, J.-P. (1975). Changes of pituitary sensitivity to LH-RH during the rat estrous cycle. *Molecular and Cellular Endocrinology* 2, 107-115.

- Fink, G. (1976). Control of the rat estrous cycle. Proceedings of the Vth International Congress of Endocrinology ; Amsterdam ; Excerpta Medica. In press.
- Hobson, W. and Hansel, W. (1974). Increased in vitro pituitary responsiveness to LH-RH after in vivo estrogen treatment. Proceedings of the Society for Experimental Biology and Medicine 146, 470-474.
- Kalra, S.P. (1975). Observation of facilitation of the preovulatory rise of LH by estrogen. Endocrinology 96, 23-28.
- Labrie, F. (1976). Mechanism of action of hypophysiotropic hormones. Proceedings of the Vth International Congress of Endocrinology ; Amsterdam ; Excerpta Medica. In press.
- Legan, S.J. and Karsch, F.J. (1975). Modulation of pituitary responsiveness to luteinizing hormone-releasing factor during the estrous cycle of the rat. Endocrinology 96, 571-575.
- Parker Jr., C.R., Costoff, A., Muldoon, T.G. and Mahesh, V.B. (1976). Action of pregnant mare serum gonadotropin in the immature female rat : correlative changes in blood steroids, gonadotropins, and cytoplasmic estradiol receptors of the anterior pituitary and hypothalamus. Endocrinology 98, 129-138.
- Ruf, K.B., YoungLai, E.V. and Holmes, M.J. (1974). Induction of precocious sexual maturation in female rats by electrochemical stimulation of the brain. Brain Research 78, 437-446.
- Vilchez-Martinez, J.A., Arimura, A., Debeljuk, L. and Schally, A.V. (1974). Biphasic effect of estradiol benzoate on the pituitary responsiveness to LH-RH. Endocrinology 94, 1300-1303.
- Wilkinson, M., de Ziegler, D. and Ruf, K.B. (1976). Facilitatory effect of oestradiol on luteinizing hormone secretion from immature female rat pituitaries in vitro. Journal of Endocrinology 70, 155-156.
- Wilson, C.A., Horth, C.E., Endersby, C.A. and McDonald, P.G. (1974). Changes in plasma levels of oestradiol, progesterone and luteinizing hormone in immature rats treated with pregnant mare serum gonadotrophin. Journal of Endocrinology 60, 293-304.
- YoungLai, E.V., Holmes, M.J. and Ruf, K.B. (1976). Changes in concentration of LH, FSH and estrogen in the immature female rat during precocious sexual maturation induced by electrochemical stimulation of the brain. Hormone Research, in press.
- Zeballos, G. and McCann, S.M. (1974). The effect of subcutaneous administration of synthetic luteinizing hormone-releasing factor on plasma gonadotropins and prolactin in the rat. Proceedings of the Society for Experimental Biology and Medicine 145, 415-420.
- Zeballos, G. and McCann, S.M. (1975). Alteration during the estrous cycle in the responsiveness of the pituitary to subcutaneous administration of synthetic LH-releasing hormone (LHRH). Endocrinology 96, 1377-1385.

de Ziegler, D. , Wilkinson, M. and Ruf, K.B. (1976). Precocious puberty in female rats : on the mode of action of hypothalamic lesions. Annales de Biologie animale, Biochimie, Biophysique 16, 443-451.

Table 1

Controls	-/OB	OB/-	OB/OB	OB/P		PMSG
40 ± 2	82 ± 4	121 ± 3	136 ± 4	118 ± 3	80 ± 9	137 ± 4
(24)	(20)	(18)	(32)	(20)	(29)	(44)

Uterine weight (mg ± S. E. M.) after vehicle injection (controls), one injection of OB on day 25 (-/OB), or day 23 (OB/-), two injections of OB on day 23 and day 25 (OB/OB), one injection of OB on day 23 and one injection of P at 1200 h on day 23 (OB/P), brain lesion () placed on day 23, or 10 i. u. PMSG administered on day 24 (PMSG). Numbers in brackets indicate number of animals. All results are statistically significantly different from control ($p < 0.001$).

Table 2

Age in days :	23	24	25	26	27
♂		51 ± 22	47 ± 7	41 ± 4	53 ± 9
		370 ± 75	276 ± 56	250 ± 61	524 ± 42
		(5)	(5)	(5)	(5)
C	32 ± 2	57 ± 5	50 ± 21	43 ± 14	61 ± 29
	862 ± 11	460 ± 85	425 ± 37	334 ± 55	254 ± 28
	(5)	(5)	(5)	(5)	(5)

Plasma LH values (ng/ml ; ± S. E. M.) obtained before (upper line) and after (lower line) 100 ng GnRH (i. v.) in female rats receiving brain lesion on day 23 (♂) (upper row) or sham-operated (C) (lower row). Numbers in brackets indicate number of animals.

Table 3

Plasma LH (ng/ml)		C	-/OB	OB/OB
Basal values				
	1000 h	20 ± 5 (6)	24 ± 3 (6)	22 ± 2 (8)
	1400 h	22 ± 2 (8)	19 ± 4 (7)	21 ± 3 (8)
	1700 h	21 ± 2 (6)	23 ± 5 (6)	107 ± 33 (6)
One injection (30 min)				
	1000 h	41.9 ± 7.7 (6)	8.91 ± 9.9 (10)	91.6 ± 11.2 (7)
	1400 h	44.9 ± 8.4 (7)	8.08 ± 8.8 (8)	125.9 ± 18.4 (12)
	1700 h	41.8 ± 4.0 (5)	10.87 ± 3.40 (6)	123.5 ± 13.8 (6)
One injection (90 min)				
	1000 h	7.2 ± 1.2 (6)	8.4 ± 1.1 (11)	10.9 ± 1.5 (7)
	1400 h	6.7 ± .9 (6)	1.55 ± .28 (6)	2.83 ± .42 (6)
	1700 h	4.1 ± .8 (6)	8.0 ± 2.0 (6)	9.08 ± 2.63 (6)
Two injections (30 min)				
	1000 h	6.21 ± .40 (16)	18.19 ± 1.83 (13)	18.25 ± 3.43 (14)
	1400 h	9.40 ± .50 (6)	1.970 ± 1.00 (6)	2.644 ± .472 (6)
	1700 h	8.64 ± 1.05 (6)	18.56 ± 2.59 (6)	31.25 ± 2.55 (6)

Plasma LH values (ng/ml ; ± S.E.M.) ; (a) basal values ; (b) 30 min ; and (c) 90 min after one injection of GnRH, and (d) 30 min after two GnRH injections separated by a 60 min interval at 1000, 1400 and 1700 h in 26 day-old female rats treated with either vehicle only (C), one injection of OB on day 25 (-/OB), or two injections of OB on days 23 and 25 (OB/OB). Numbers in brackets indicate number of animals. Increments observed in -/OB and OB/OB animals after one and two injections of GnRH are significantly greater than those of C ($p < 0.01$). Responsiveness in the OB/OB group exceeds ($p < 0.01$) that of -/OB group when two GnRH injections are administered at 1700 h on day 26. Numbers in brackets indicate number of animals.

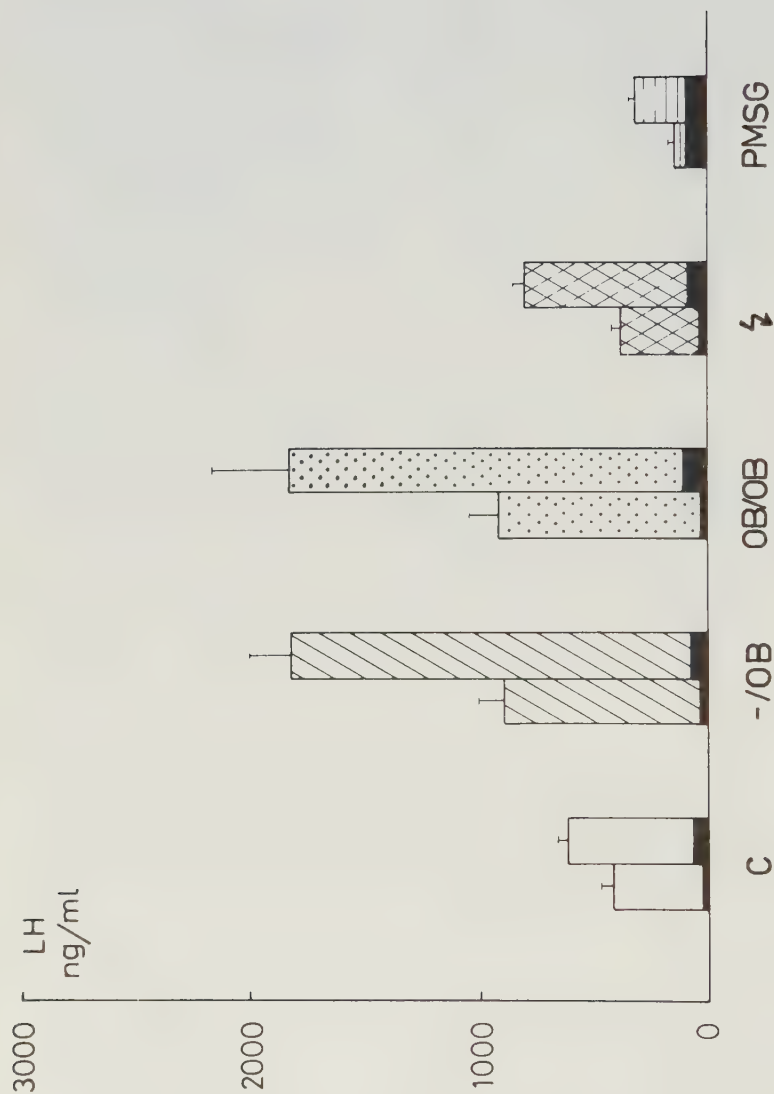


Fig. 1a :

Plasma LH (ng/ml ; $\pm$ S.E.M.) observed 30 min after one (left hand) and two GnRH injections (right hand of paired columns) given to 26 day-old female rats primed with either one injection of OB on day 25 (-/OB) , 2 injections of OB on days 23 and 25 (OB/OB) , brain lesion carried out on day 23 ($\frac{1}{4}$) , or PMSG given on day 24 ; first injection at 1000 h ; second injection 60 min after the first. Basal LH values represented by shaded area of left hand column. Shaded area of right hand column is plasma LH levels 90 min after first GnRH stimulation alone.

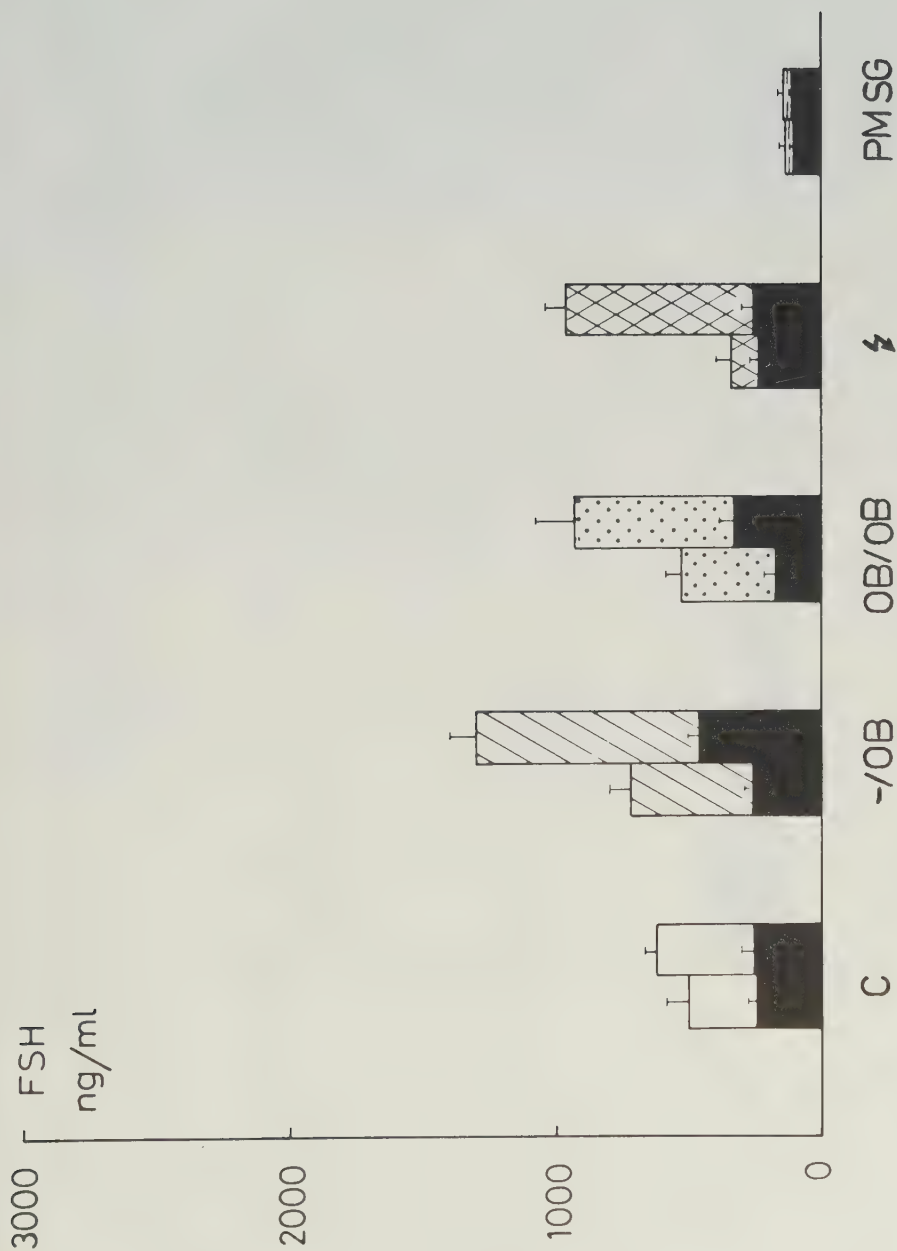


Fig. 1b : Plasma FSH values, obtained in same groups as described in Fig. 1a.

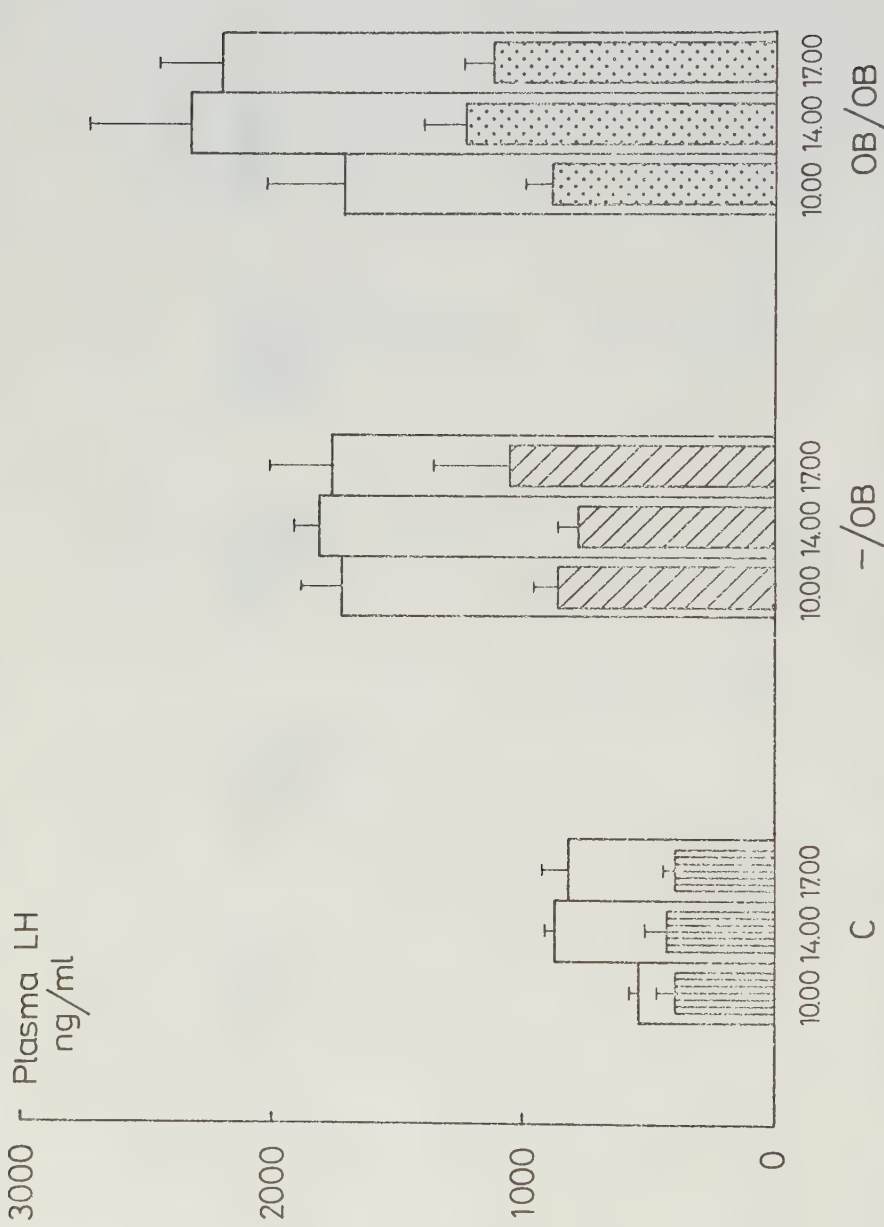


Fig. 2 Increments in plasma LH (ng/ml ; + S. E. M.) 30 min after one (inner columns) and two (outer columns) in 26 day -old female rats at 1000 h (left column, each group), 1400 h (middle column) and 1700 h (right column) primed with one injection of OB on day 25 (-/OB) , two injections of OB on days 23 and 25 (OB/OB) , or vehicle only (C) .

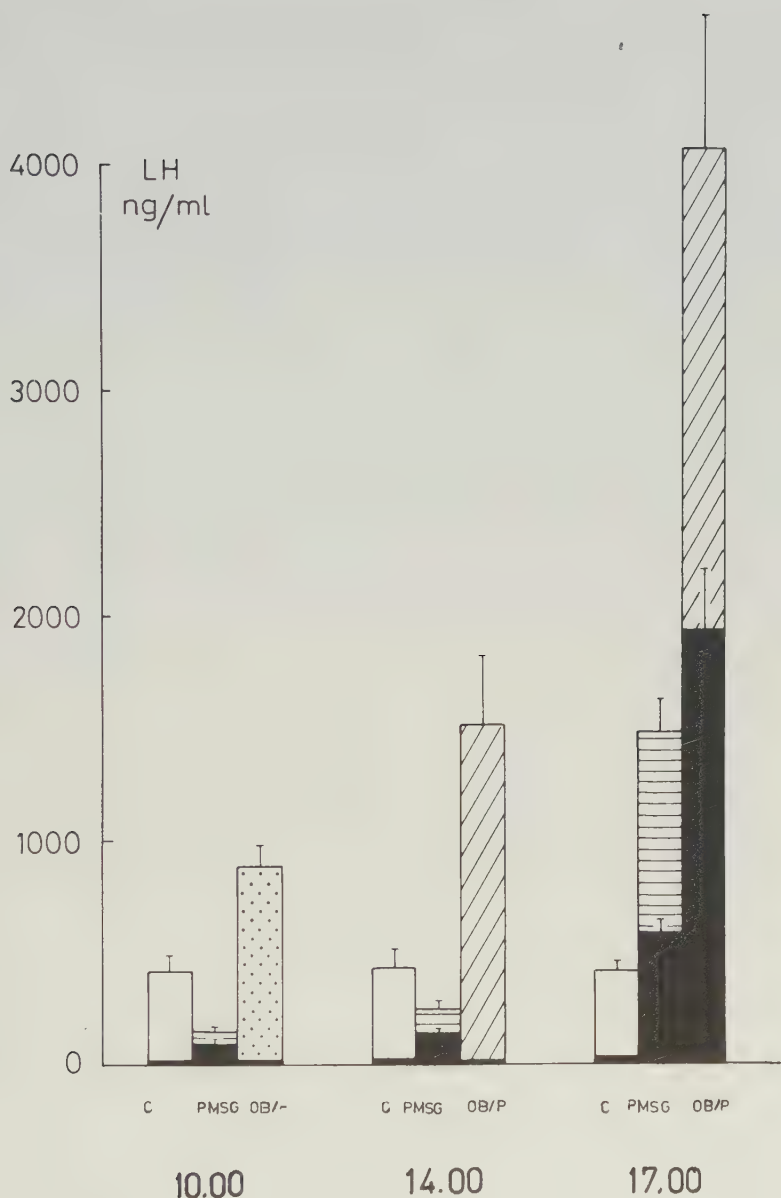


Fig. 3

Plasma LH (ng/ml ; $\pm$ S.E.M.) obtained 30 min after one injection of GnRH in 26 day-old female rats primed with either vehicle (C) $\square$, PMSG on day 24 $\square$, one injection of OB on day 23 (OB/-) $\square$, or one injection of OB on day 23 and one injection of P on day 26 (OB/P) $\square$. Basal values in animals of same age, similarly primed, but without GnRH, given by shaded bars. Experiment repeated at 3 different times, day 26 : 1000, 1400 and 1700 h.

CHAPTER IV

CONCLUSIONS AND COMMENTS

IV. CONCLUSIONS AND COMMENTS

The influence of oestrogen on adeno-hypophysial sensitivity to stimulation with GnRH was investigated in the immature female rat in a variety of experimental conditions. Both *in vitro* (chapter II) and *in vivo* (chapter III) techniques were used. The results obtained allow us to draw the following conclusions :

- 1) Both the *in vitro* and *in vivo* techniques used are adequate for the study of the given problem and yield identical results.
- 2) Pretreatment of immature female rats with oestradiol benzoate (OB) increases the pituitary sensitivity to GnRH stimulation as described in adult females (105)*. This effect has been observed at all ages tested *in vitro* and is thus present as early as day 5 of life. This is considerably earlier than the age at which oestrogen is able to induce positive (stimulatory) feedback effects in terms of a gonadotrophin surge (about 21 days of age) (36, 145). We conclude, therefore, that the induction of a gonadotrophin surge by oestrogen must involve maturation of mechanisms operative in addition to the enhancement of pituitary responsiveness (e.g. direct effect of oestrogen on hypothalamic GnRH secretion and/or induction by oestrogen of the so-called "self-priming" effect of GnRH ?).
- 3) A single injection of oestrogen-benzoate administered on day 25 of life is sufficient to enhance pituitary sensitivity tested on day 26. This enhancement of pituitary sensitivity is not further increased by a more prolonged treatment consisting of two injections of OB applied on days 23 and 25 despite a significantly ($p = 0.001$) more pronounced increase in uterine weight observed in the latter case.
- 4) Immature female rats treated with pregnant mare serum gonadotrophin (PMSG) on day 24 exhibit a reduced sensitivity to GnRH when tested at 1000 and 1400 h on the day of induced pro-oestrus (day 26) as compared to that observed in unprimed controls. However, at 1700 h an LH surge is observed and pituitary sensitivity is found increased at that time, largely exceeding that of controls. This observation was unexpected and is in striking contrast to conditions prevailing, in the adult cycling rat, on the day of pro-oestrus, where a rise in pituitary responsiveness is already manifest in the morning and early afternoon i.e. before any elevation in plasma LH takes place (99). It is possible that the high plasma testosterone concentrations found in PMSG-treated animals (91) counterbalance the effect of oestrogen and decrease pituitary sensitivity to GnRH (152). We conclude from this observation that the occurrence of an oestrogen-

* cf the reference list, at the end of Chapter I (Introduction), for numbers in brackets.

induced gonadotrophin surge is not dependent on prior enhancement of pituitary sensitivity ; it can be triggered in conditions of overall-decreased pituitary responsiveness as well. The increased response, seen in PMSG-treated animals at the height of the gonadotrophin surge, is probably the result of the "self-priming" effect of GnRH (which increases the responsiveness of the gland to further stimulation) (108). In the adult cycling rat, this self-priming effect is only demonstrable on the day of pro-oestrus and was thus believed to be linked to the oestrogen-induced increase in pituitary sensitivity. Our observations in the PMSG-treated animals indicate that these two phenomena can be dissociated as well, since the self-priming effect is not preceded by enhanced pituitary responsiveness. These differences call for caution in the indiscriminate use of the PMSG treated animals as a model of the adult oestrous cycle.

APPENDIX I

In contrast to certain other laboratory animals (e.g. rabbits) the female rat ovulates spontaneously and its reproductive cycle (oestrous cycle) is extremely short, lasting 4 to 5 days depending on individuals and strains. Ovulation is connected with the lighting schedule and regularly occurs during the early hours of oestrus (0200 h). A rise in ovarian steroids as well as in gonadotrophins is observed on the day which preceeds ovulation, i.e. pro-oestrus. A strikingly sharp surge of LH is observed during the afternoon of pro-oestrus, around 1700 h. An elevation in plasma FSH is also observed on pro-oestrus but in a less dramatic way and later during the evening (2300 h) (see Fig. 4). (For more details, see for example ref. 1, 73*).

Recently, an elevation of pituitary stalk blood content in radio-immuno-assayable GnRH has been observed both around 1700 h and 2300 h (109).

Fig. 2 illustrates the oestrogen secretion rate through the oestrous cycle. Elevation in plasma oestradiol is maximum on the orning of pro-oestrus (15). Plasma progesterone sharply increases during the afternoon of pro-oestrus (16).

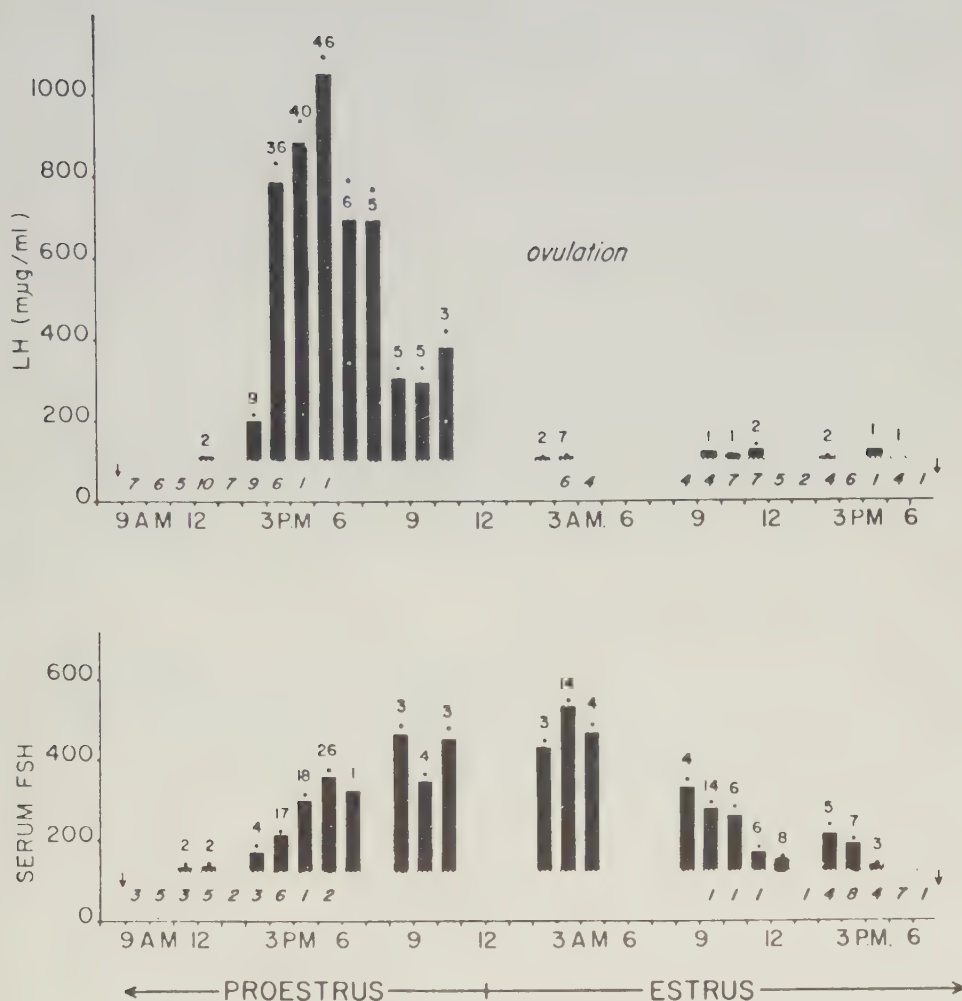


Fig. 4 Concentration of LH and FSH in rat sera during pro-oestrous stages of the normal cycle expressed in terms of niamd-rat LH-RP-1 and niamd-rat FSH-RP-1/ml.

from Daani and Parlow, *Endocrinology* 88, 653, 1971.

